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REUSABLE LIGHT-ACTIVATED ANTIMICROBIAL MATERIALS PREPARED
WITH FOOD INGREDIENTS

by

Andrew T. Gagon

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Science

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2020

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ABSTRACT

Reusable Light Activated Antimicrobial Materials Prepared with Food Ingredients

by

Andrew Thomas Gagon, Master of Science

Utah State University, 2020

Major Professor: Dr. Luis J. Bastarrachea
Department: Nutrition and Food Science

Chitosan is a polycationic structure and an inherent antimicrobial compound. It has been a topic of study as an antimicrobial for a variety of industries. Zein is a protein found in the endosperm of corn kernels, but does not possess any intrinsic antimicrobial characteristics. It is still used for different purposes due to its multiple functionalities. In this study, antimicrobial plastics were developed through modifying polypropylene to harbor chitosan or zein. These antimicrobial plastics were challenged against *Escherichia coli* K12 while simultaneously being exposed to UV-A light. The materials proved to be effective in inhibiting growth of the test organism for 10 cycles, providing between ~ 90 – 94 % reduction or between 1-2 log(CFU/mL) reduction across 10 cycles of continuous reuse. Fluorometric evaluations suggested that reactive oxygen species are being generated and are the driving factor in biocidal effect. Infrared spectroscopy and electron microscopy confirmed marginal change occurred to the surface and confirmed the stability of the materials. In addition, electron microscopy analysis on the bacteria exposed to this material under UV-A suggested damage to cell morphology.

(91 pages)

PUBLIC ABSTRACT

Reusable Light-Activated Antimicrobial Materials Prepared with Food Ingredients

Andrew Thomas Gagon

Microbial contamination and proliferation in food processing, distribution and storage continues to be a real risk in spite the wide variety of food processing and preservation techniques available. Some of these techniques (such as heat treatments and the use of chemical preservatives) may require substantial amounts of energy and their effects in the general population and the environment may be harmful. This has generated an interest over alternative methods of food preservation. The use of reusable light-activated materials may represent an effective approach. In this study, a polypropylene plastic was modified with either zein, which is found in corn, or chitosan, which can be found in the shell of crustacea, and tested it against *E. coli* K12 while also being exposed to UV-A light. Both materials displayed abilities to inactivate the bacteria by up to 94% over 10 cycles (1 cycle including contact with aqueous *E. coli* K12 and 30 min of UV-A light). Analysis of the surface through microscopic and infrared spectroscopy showed minor change in the materials. These materials have shown promise in inhibiting growth of bacteria, particularly in aqueous environments.

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LIST OF ABBREVIATIONS

Acid Orange 7 (AO7)
Analysis of Variance (ANOVA)
Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)
Atomic Force Microscopy (AFM)
Deionized (DI)
 ϵ -Poly(lysine) (EPL)
Escherichia coli (*E. coli*)
Ethylenediamine tetraacetic acid (EDTA)
Generally Recognized As Safe (GRAS)
Lauric Acid (LA)
Methyl Vinyl Ether Maleic Anhydride copolymer (MVE)
Peptone Water (PW)
Polypropylene (PP)
Polypropylene/Polypropylene-*graft*-Maleic Anhydride (PP-MA)
Polypropylene-Methyl Vinyl Ether-Chitosan (PP-MVE-CHI)
Polypropylene-*graft*-Zein (PP-Z)
Reactive Oxygen Species (ROS)
Scanning Electron Microscopy (SEM)
Tryptic Soy Agar (TSA)
Tryptic Soy Broth (TSB)
Ultraviolet Light (UV)

CHAPTER 1

INTRODUCTION

Traditional Methods of Food Preservation

In 2008, it was estimated that food production must increase approximately seventy percent by 2050 in order to maintain the needs of the world population (Floros et al., 2010). This has inspired considerable advancement in the field of food production, particularly in development of technology. As technology has advanced so has the production of food. During food production, the main objectives are preventing spoilage and, at the same time, ensuring it is safe and nutritious. However, spoilage can occur through means of chemical processes like enzymatic reactions, physical changes, and most commonly through microbial means (Potter & Hotchkiss, 1998). Preserving food through various methods of processing allows producers to prevent or delay spoilage and may enhance the palatability as well. Understanding different means of preserving food is fundamental to ensure long shelf stability and high quality food.

Food preservation is defined as techniques used to minimize internal and external risk factors that may cause food spoilage (Amit, Uddin, Rahman, Islam, & Khan, 2017). There are diverse methods for food preservation. Among the most widely used techniques are refrigeration and freezing, dehydration, acidification, fermentation, water activity control, and use of additives (Vaclavik & Christian, 2014). Freezing allows for the water activity to be reduced to a point where microorganisms cannot multiply (Speck & Ray, 1977). Drying has been stated as one of the oldest and most common techniques for food preservation (Mustapha & Lee, 2017; Sharif, Mohd Yusof, Zaki, Mustapha, & Jai, 2017). Drying will remove water from the food, reducing water activity and the opportunity for

microorganisms to multiply. These are all techniques that are being used today to increase the shelf-life of foods. The wide use of these techniques of food preservation may be attributed to their effectiveness, reliability and predictability. However, these techniques also have disadvantages such as detrimental effects on the quality attributes of foods, cross contamination (such as with refrigeration), and recrystallization (Vaclavik & Christian, 2014). This has raised interest in alternative techniques such as microwave heating, ohmic heating, high pressure processing, pulsed electric fields, and ultraviolet (UV) light (Floros et al., 2010).

Use of Ultraviolet Light

UV light has been of interest as an alternative preservation technique. Some of the reasons UV light is being studied is that it may be able to treat foods without reducing the quality as compared to traditional methods, its high throughput, and continuous operation (Tikekar, LaBorde, & Anantheswaran, 2010). This may also satisfy consumers who are looking for “greener” alternatives, as compared to foods with chemical preservatives (Koutchma, 2009).

UV light is divided into 3 different ranges of wavelengths. The UV-A interval (315 – 400 nm) can cause skin tanning and aging, the UV-B interval (280 – 315 nm) is known to cause skin cancer, and the UV-C interval (200 – 280 nm) which is lethal to microorganisms (Keklik, Krishnamurthy, & Demirci, 2012; Tikekar et al., 2010). When UV-C light is absorbed by the DNA of microbial cells, cross-linking occurs in cytosine or thymine residues on the same strand. This in turn restricts microbial cells from reproducing, causing their death. Due to its low maintenance requirements and lower energy costs, UV-

C light can be an effective alternative (Guerrero-Beltrán & Barbosa-Cánovas, 2004). However, UV-B and UV-C light are dangerous as they may cause skin cancer in humans (Pfeifer & Besaratinia, 2012). Due to this potential hazard, UV-A light, which could have lower potential for such risks, may be able to inactivate microorganisms in combination with light-activated materials without the harm to humans (Pfeifer & Besaratinia, 2012; Tikekar et al., 2010).

The main uses for UV light focus on surface and equipment disinfection and water treatment (Keklik et al., 2012). It is also being used to treat packaging materials that cannot withstand the traditional thermal treatment (Koutchma, 2008). Although this is a viable alternative to thermal treatment, there are still some disadvantages to using UV light. The difficulty of using UV light on foods is that its penetration diminishes within a medium, which in turn attenuates any biocidal effect (Gouma, Gayán, Raso, Condón, & Álvarez, 2015; Koutchma, 2008). The Lambert-Beer law explains the attenuation effect of UV light as follows:

$$I = I_0 e^{(\alpha d)}$$

where I is the UV intensity at depth d of a medium, I_0 relates to the lamp UV intensity incident at the surface of the medium, and α refers to the medium absorption coefficient (Lopez-Malo & Palou, 2005). Nevertheless, UV light has shown promise despite this drawback.

Light-Activated Compounds and Materials

The use of UV light has also been explored in combination with light-activated or photoactive compounds. Light-activated compounds are defined as compounds that exhibit

antimicrobial activity when exposed to a specific type of light. Their biocidal properties are due to the result of Reactive Oxygen Species (ROS) generation (Figure 1.1), which then react with the cell membranes and other biomolecules (e.g. enzymes or nucleic acids). Photoactive compounds that have been used with light-activation include porphyrin, chlorophyll derivatives, TiO_2 , and polycations (Bastarrachea, Denis-Rohr, & Goddard, 2015).

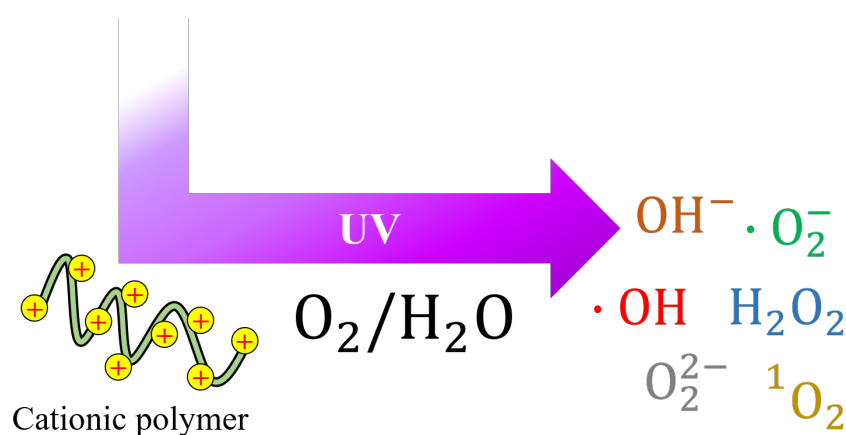


Figure 1.1 Reactive Oxygen Species being generated after light-activated compounds are exposed to specific wavelengths of light

Different light-activated materials have demonstrated efficacy against Gram-positive and Gram-negative bacteria under experimental conditions. Some examples of materials in which light-activated compounds have been incorporated are polysiloxane, cellulose acetate, or stainless steel. However, this concept presents some drawbacks that may limit its application in food systems, such as limited effectiveness in the presence of organic matter and low chemical and physical stability of the modified materials (Bastarrachea, Denis-Rohr, et al., 2015).

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CHAPTER 2

LITERATURE REVIEW

Antimicrobial Polycations

Polycations are an example of compounds used to inhibit microbial growth. This type of compound has multiple positive charges (Deka, Sharma, & Kumar, 2015). Chitosan (Figure 2.1) is a polycation derivative of chitin, which can be found in plants and also in the shell of crustaceans (Goy, Britto, & Assis, 2009). Chitosan has been researched extensively due to its natural ability to hinder microbial growth and its low toxicity to humans (Verlee, Mincke, & Stevens, 2017). The mechanism that allows chitosan to inactivate bacteria is believed to involve electrostatic interactions with the anionic teichoic acids found on the cell wall of Gram-positive cells, which prevents them from reproducing and surviving (Verlee et al., 2017). Gram-negative bacterial cells are also believed to interact with chitosan in a similar manner (Goy et al., 2009). A study was conducted to determine whether the molecular weight of chitosan would be a factor in its antimicrobial abilities (Zheng & Zhu, 2003). The study challenged varying concentrations of chitosan at different molecular weights against *Escherichia coli* and *Staphylococcus aureus* by plating the chitosan on the test culture. They found that a 1% concentration of chitosan at any molecular weight was able to inhibit the growth of both cultures by 100%. It also showed that increasing the molecular weight decreased the antimicrobial ability against *E. coli* and increased it against *S. aureus*. More recently, sulfonated chitosan was challenged against *E. coli* and *S. aureus* in a study done by Sun, Shi, Wang, Fang, and Huang (2017). It was found that the sulfonated chitosan had a minimum inhibitory concentration of 0.13 mg/mL

for *E. coli* and 2.00 mg/mL for *S. aureus*, while water soluble chitosan had a minimum inhibitory concentration of 0.5 mg/mL and 4.00 mg/mL for *E. coli* and *S. aureus*, respectively.

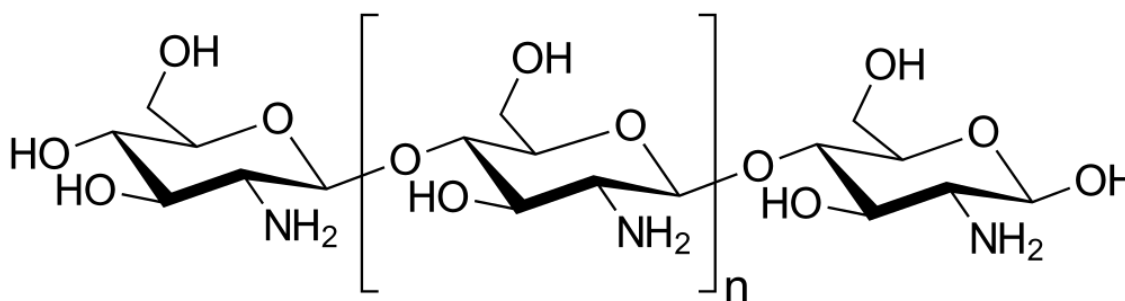


Figure 2.1 Chemical structure of chitosan

Another example of an antimicrobial polycation is ϵ -poly(lysine) (EPL). This agent is a polypeptide that has been given the status of generally recognized as safe (GRAS) and has been shown to be effective as a food preservative (Kozak, Brown, Bobak, & D'Amico, 2017). Kozak et al. tested this compound against *Listeria monocytogenes* in milk to determine its efficacy in a fluid food. It was found that with at concentration greater than 100 mg/L, EPL inhibited the growth of *L. monocytogenes* by approximately 4 logarithmic reductions after day 21 of storage (2017).

Antimicrobial Synergy between UV Light and Polycations

A recent study found that UV-A light promotes a biocidal effect in conjunction with certain food ingredients (Bastarrachea, 2019). This study reported that EPL, when exposed to UV-visible light, generated ROS. Further investigation found that the polycation EPL, which is intrinsically antimicrobial, when exposed to UV-A light may have had an enhanced antimicrobial activity when challenged against *E. coli* K12. It was shown that the UV-A

light generated ROS within the EPL when exposed for 15 min at a distance of 15 cm, thereby increasing the biocidal effectiveness (Bastarrachea, 2019).

Ji, Corbitt, Parthasarathy, Schanze, and Whitten determined the mechanism of cationic conjugated polyelectrolytes as light-activated compounds to be singlet oxygen (a reactive oxygen species) being generated, which ultimately alters the microorganisms' cellular functions (2011). In one of their studies, Ji et al. functionalized poly(phenylene ethynylene) with varying degrees of polymerization and challenged it against *E. coli* and *Staphylococcus epidermidis* under UV-A light. It was observed that all degrees of polymerization were able to kill ~ 99% of *E. coli* cells after 120 min of UV-A light exposure and had nearly no effect when left in the dark after the same amount of time. They also found the cation to be more effective when challenged against *S. epidermidis*, where ~ 99% were killed after 30 min of UV-A exposure (Ji, Parthasarathy, Corbitt, Schanze, & Whitten, 2011). A different study was done where the material polyamide 56 was modified with Reactive Blue P-3R and 3,3',4,4'-Benzophenone tetracarboxylic acid and challenged against *S. aureus* and *E. coli*. It was found that when this modified material was exposed to UV-A light, it generated hydroxyl radicals, which lead to bacterial cell death (Gao, Zhang, Sun, Xie, & Hou, 2017).

Use of Zein

Zein is a protein that can be found in the endosperm of corn. It has an isoelectric point at pH 6.2 and a molecular weight between 9.6 and 44 kDa (Rishi & Munir, 2001). Proteins and peptides have been used to modify materials to render them antimicrobial (Bastarrachea, Denis-Rohr, et al., 2015). Their antimicrobial characteristics are due to their

positive charge that interact with bacteria in a similar fashion to polycations (Bahar & Ren, 2013). Zein is a unique protein in that it has not been reported to have an intrinsic antimicrobial properties and it is hydrophobic, thus leading to poor solubility in water and improved solubility in alcohol (Rishi & Munir, 2001). This alcohol solubility may provide better barrier properties (Tihminlioglu, Atik, & Özen, 2010). Despite the lack of inherent antimicrobial characteristics, it has been used in other types of food applications, such as encapsulation of drugs and pesticides, and biodegradable packaging (Sengupta & Han, 2013). One such study challenged zein impregnated with either Ethylenediamine tetraacetic acid (EDTA), nisin, or lauric acid (LA) against *L. monocytogenes* and *Salmonella enteritidis*. The study also analyzed combinations of EDTA and LA, EDTA and nisin, and a combination of all 3. A zein film was prepared using a casting method. The antimicrobial agents were added just before the casting step. It was found that EDTA in the zein film had no effect on *L. monocytogenes*, but LA was able to reduce the population by 5 logarithmic cycles after 48 h. The nisin was also able to reduce the population by 5.5 logarithmic cycles after 48 h (Hoffman, Han, & Dawson, 2016). Another study infused zein with thymol or carvacrol and challenged the film against a nonpathogenic strain of *E. coli*. The film was able to decrease growth of *E. coli* by up to 1.8 log(CFU/mL) and zein was able to enhance the solubility of the essential oils by up to 15 fold (Wu, Luo, & Wang, 2012).

Antimicrobial Materials

Antimicrobial materials are a means of preventing microbial growth. Several methods are available for material modification to render them antimicrobial (Bastarrachea, Denis-

Rohr, et al., 2015). Methods described by Bastarrachea et al. include graft polymerization, crosslinking, layer-by-layer coating, self-assembled monolayers, chemical vaporization, and electroless and electroplating (2015). Another method of material modification is through noncovalent bonding, where adsorption of a compound of interest to the surface of a material is due to electrostatic interactions or ligand-receptor pairing, and covalent bonding, where a stable bond between a compound and a surface may be formed (Goddard & Hotchkiss, 2007). A study modified NH₂ glass slides by covalently attaching poly(4-vinyl-N-alkylpyridinium bromide) and assessed its antimicrobial activity against airborne *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *E. coli*. The study concluded that the slides were able to inhibit growth of each bacteria between 94 to 99% (Tiller, Liao, Lewis, & Klibanov, 2001).

Each of these methods has its advantages and disadvantages, especially depending on the purpose of the material, which will be discussed further in the section below. Covalent bonding allows for a molecule to stay attached on the surface of the material, which prevents it from separating out to the food substance. Noncovalent bonding may be used for controlled release of compounds (Goddard & Hotchkiss, 2007).

Difficulty of Reusability

Even though the concept of antimicrobial materials seems to be an attractive alternative to other traditional methods of food preservation, there is still a disadvantage: these materials may lose their efficacy over time (Bastarrachea, Denis-Rohr, et al., 2015). This was confirmed in a study by Cowan, Abshire, Houk, and Evans (2003). In the study, stainless steel was modified to harbor zinc and silver nanoparticles and tested against *E. coli*, *S.*

aureus, *Pseudomonas aeruginosa*, and *L. monocytogenes*. They observed that the modified steel was able to reduce growth between 4 and 6 logarithmic cycles, but after cleaning the steel and testing again, the reduction dropped to between 15 and 86%. Another disadvantage is that as the moiety is attached to the surface it loses some of its functionality (Bastarrachea, Denis-Rohr, et al., 2015). It is also apparent that some of these reactive groups lose their activity with repeated exposure to organic materials (Bastarrachea, 2019; Bastarrachea & Goddard, 2016). Many studies have shown that antimicrobial coatings are effective in laboratory conditions, but their application in more realistic conditions like the ones found in industry seems limited (Bastarrachea, 2019).

Reactive Blending

Reactive blending is a technique in which two or more substances are fused together through the application of heat, and in this process covalent bonds between the different substances can be formed. A type of functional group used to promote cross-linking in reactive blending is cyclic anhydrides, which can form covalent bonds with amine and hydroxyl groups. Polyanhydrides are molecules that have an anhydride group either in the backbone or in a side group (N. Kumar, Langer, & Domb, 2002). In recent works, polycations have been covalently attached onto the surface of anhydride-containing polymer plastics through reactive blending. Bastarrachea and Goddard (2016) developed an antimicrobial plastic made with an anhydride incorporated polypropylene and polyethyleneimine. This material was able to reduce the microbial load of aqueous suspensions with *E. coli* O157:H7 by $\sim 3 \log(\text{CFU/mL})$. In addition, the antimicrobial plastic retained antimicrobial efficacy in the presence of organic matter and displayed self-

healing properties. This latter property was confirmed by exposing the material to extreme levels of pH and subjecting it to reheating, which resulted in the restoration of the bonds that held together the bulk polypropylene and the polycation. In a more recent study, Bastarrachea (2019) followed a similar reactive blending protocol to prepare an antimicrobial material consisting of anhydride-containing polypropylene and ϵ -poly(lysine). In this case, the polycation also formed covalent bonds with the polypropylene support through the formation of imide bonds upon heating (185 °C for 1 h). The biocidal efficacy was confirmed against *E. coli* K12 with or without UV-A light exposure, both in aqueous suspensions (> 4 logarithmic reductions) and in apple juice (~ 1 logarithmic reduction). Fluorometric assays confirmed that part of the mechanisms behind the antimicrobial effectiveness was ROS generation, specifically singlet oxygen.

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HYPOTHESIS AND OBJECTIVES

Plastic supports, such as polypropylene, can be modified through reactive blending to harbor substances or compounds that are antimicrobial. These polypropylene films will be coated with either chitosan or zein. The combination of UV-A light and antimicrobial materials may result in an effective alternative to reduce the microbial load in foods. The use of reactive blending can also result in robust and stable materials that can be reutilized.

Objective 1

A chitosan-modified plastic will be developed through reactive blending of anhydride modified polypropylene and the polycation. The material containing chitosan will be characterized through surface analysis techniques and its antimicrobial effectiveness and reusability will be tested for at least 10 cycles.

Objective 2

A zein-modified plastic will be created through reactive blending of an anhydride modified polypropylene and the protein. The material containing zein will be characterized through surface analysis techniques and tested for its antimicrobial effectiveness and reusability for at least 10 cycles.

CHAPTER 3

ANTIMICROBIAL LIGHT-ACTIVATED POLYPROPYLENE MODIFIED WITH CHITOSAN: CHARACTERIZATION AND REUSABILITY¹

ABSTRACT

Chitosan is a polycationic structure and an inherent antimicrobial compound. Through reactive blending, a robust and stable light-activated antimicrobial plastic that is also reusable was prepared. The antimicrobial plastic (PP-MVE-CHI) was challenged against *Escherichia coli* K12 while simultaneously being exposed to UV-A light for 30 min. The antimicrobial plastic retained its antimicrobial efficacy for 10 cycles, providing $94.0 \pm 3.3\%$ reduction in the microbial population across the 10 cycles. Infrared spectroscopy confirmed marginal effect on the surface chemistry after the 10 cycles. Fluorometric evaluations suggested that the antimicrobial effect was driven by reactive oxygen species generation. No evidence of fouling on the material was found through microscopic techniques. In addition, electron microscopy analysis on the bacteria exposed to this material under UV-A suggested damage to cell morphology.

INTRODUCTION

Food preservation methods have been used for centuries to curtail product deterioration, reduce microbial growth, and maintain overall acceptability (Mustapha & Lee, 2017).

¹ Journal of Agriculture and Food Chemistry. Antimicrobial Light-Activated Polypropylene Modified With Chitosan: Characterization and Reusability. December 2019. Andrew T. Gagon, David W. Britt, Luis J. Bastarrachea. 10.1021/acs.jafc.9b06009. (original copyright notice as given in the publication in which the material was originally published) "Reprinted (adapted) with permission from Antimicrobial Light-Activated Polypropylene Modified With Chitosan: Characterization and Reusability. Copyright 2019 American Chemical Society".

Some of these methods may include heating, dehydration, refrigeration, freezing, acidification, fermentation, water activity control, smoking, chemical preservatives, irradiation, and modified or controlled atmosphere storage (Floros et al., 2010). These techniques, although effective, may have some drawbacks. They may decrease the nutritional quality of foods (Sharif et al., 2017), or may require high energy consumption (Rodriguez-Gonzalez, Buckow, Koutchma, & Balasubramaniam, 2015). These types of drawbacks have led to the exploration of alternative methods for preservation such as natural preservatives (Tiwari et al., 2009), pulsed electric fields, high hydrostatic pressures, ultrasound, ultraviolet (UV) light (Aneja, Dhiman, Aggarwal, & Aneja, 2014), and antimicrobial materials (Bastarrachea, Denis-Rohr, et al., 2015).

Commonly used materials may be modified to possess antimicrobial properties. Methods to achieve this include layer-by-layer deposition, self-assembled monolayers, polymer photo grafting, surface immobilization, and embedding of active agents (Bastarrachea, Wong, Roman, Lin, & Goddard, 2015). A variety of antimicrobial compounds have been used along with these techniques to prepare antimicrobial materials. Compounds that have received attention are metal-based materials such as silver nanoparticles, quaternary ammonium compounds, antimicrobial peptides, essential oils, cationic polymers, and light-activated compounds (Bastarrachea, Denis-Rohr, et al., 2015). Light-activated compounds are chemical substances that, when exposed to a specific wavelength of light, generate Reactive Oxygen Species (ROS), which in turn can damage the cell membrane structure, enzymes, and/or nucleic acids (Bastarrachea, Denis-Rohr, et al., 2015; Huang, 2005). Compounds such as TiO_2 (Benabbou, Derriche, Felix, Lejeune, & Guillard, 2007; Yu, Ho, Lin, Yip, & Wong, 2003), Methylene Blue and Au nanoparticles

(Perni et al., 2009), and Toluidine Blue O and Rose Bengal (Decraene, Pratten, & Wilson, 2008) have been used to modify silicone (Perni et al., 2009), stainless steel (Yu et al., 2003), and glass (Decraene et al., 2008), and when exposed to the corresponding wavelength of light, they have provided 0.5 – 3.5 logarithmic reductions.

Polycations are another group of compounds that exhibit an inherent antimicrobial behavior. These compounds have also been used to modify materials in order to render them antimicrobial (Bastarrachea, Denis-Rohr, et al., 2015). Polycations interact with bacterial cells due to their positive charge, which displaces the Ca^{2+} ions attached to the cell membranes leading to loss of fluidity (Deka et al., 2015). Compounds that have been studied include poly(lysine), polyethyleneimine, polyamidoamine, and chitosan (Bastarrachea, Denis-Rohr, et al., 2015). Polycations have also shown to be photoactive upon exposure to certain wavelengths of light, being able to produce ROS, which has been attributed to their ability to harbor a positive charge (Ji, Corbitt, et al., 2011).

Chitin is a natural compound that can be found in the shell of crustacea and may be produced by fungi (Verlee et al., 2017). Chitosan is a derivative of chitin which is obtained by the partial deacetylation of chitin (Goy et al., 2009; Hamdine, Heuzey, & B  gin, 2005). It has gained interest recently due to its inherent antimicrobial properties (Kong, Chen, Xing, & Park, 2010). Chitosan is a polycation which, when exposed to acidic conditions will gain a positive charge that interacts with the negative charge of the microbial cell wall, causing cell death (Bastarrachea, Denis-Rohr, et al., 2015; S. Kumar, Ye, Dobretsov, & Dutta, 2019; Sarwar, Katas, & Zin, 2014). Due to its versatility, a variety of applications (antimicrobial and non-antimicrobial) have been explored with chitosan. It has been used in water purification to remove synthetic dyes because of the electrostatic interaction of the

hydroxyl and amino groups and due to its relative low cost (S. Kumar et al., 2019). It has also been used in the biomedical industry for tissue engineering, drug and gene delivery, wound healing, and stem cell technology due its low toxicity to cells, structural integrity, biocompatibility, biodegradability, high porosity, and predictable degradation rate (Elieh-Ali-Komi & Hamblin, 2016).

Ultraviolet light has also been a topic of study as a method to reduce or limit bacterial growth, particularly on surfaces and in water as well as a disinfection aide for pharmaceutical and electronics industries (Guerrero-Beltrán & Barbosa-Cánovas, 2004). UV light consists of three main wavelength ranges: UV-A (320 – 400 nm) which causes skin tanning and aging, UV-B (280 – 320 nm) which can cause skin cancer, and UV-C (200 – 280 nm) which is considered germicidal (Guerrero-Beltrán & Barbosa-Cánovas, 2004). UV light has also been used as a means of disinfecting surfaces of packaging and processing equipment used in meat and cheese processing facilities (Koutchma, 2008; Yang, Wu, Tai, & Sheng, 2019). The mechanism for the biocidal effect of UV light is understood to be due to dimerization of DNA. Adjacent cytosine or thymine molecules cross-link making the genetic material incapable of replication (Tikekar et al., 2010). However, UV light loses efficacy in the presence of solutes or turbidity (Koutchma, 2009). Another disadvantage is the damage UV-B and UV-C wavelengths can cause on humans (Pfeifer & Besaratinia, 2012). UV-A light may be effective in inactivating microorganisms but may require photoactivated compounds (Tikekar et al., 2010).

A disadvantage to modifying materials to harbor antimicrobial compounds is that these materials tend to be unstable against harsh environmental conditions (such as extreme temperatures or levels of pH), which limits their reusability (Bastarrachea, Denis-Rohr, et

al., 2015). Reactive blending may be used as a method to prepare stable materials by means of blending upon heating different polymers with reactive groups able to form covalent bonds, which can result in functionalized and robust materials with antimicrobial and even self-healing properties (Bastarrachea & Goddard, 2016).

In the present study, an antimicrobial plastic was prepared by modifying polypropylene for it to harbor maleic anhydride groups and by applying low molecular-weight chitosan through reactive blending. The antimicrobial plastic was also challenged against the Gram-negative bacterium *Escherichia coli* K12 ATCC 47009 with simultaneous exposure to UV-A light to assess its antimicrobial efficacy and reusability. It was then characterized through means of Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy, contact angle goniometry, Scanning Electron Microscopy (SEM), and Atomic Force Microscopy (AFM).

MATERIALS AND METHODS

Materials

Isotactic polypropylene (PP) and methyl vinyl ether/maleic anhydride copolymer (MVE) were purchased from Scientific Polymer Products (Ontario, NY, USA). Polybond 7200, a maleic anhydride-grafted polypropylene, was kindly provided by Dr. John Yun from SI group, Inc. (Niskayuna, NY, USA). Chitosan, low molecular weight (50 – 190 kDa), was from Sigma Aldrich (St. Louis, MI, USA). Acetone, absolute ethanol, sodium hydroxide, acetic acid (95%), glutaraldehyde (25% in water), and hydrochloric acid (1 N) were from Fisher Scientific (Pittsburgh, PA, USA). Anhydrous calcium sulfate was from Drierite (W. A. Hammond DRIERITE Co. LTD, Xenia, OH, USA). The dye Acid Orange 7 (AO7) was

from Electron Microscopy Sciences (Hatfield, PA, USA). Fluorescein disodium salt (376.27 g/mol) was from Acros Organics (Fair Lawn, NJ, USA). Tryptic soy agar (TSA) and tryptic soy broth (TSB) were from Difco, Becton Dickinson (Sparks, MD, USA). Peptone water (PW) was from Oxoid (Thermo Fisher Scientific, Waltham, MA, USA).

Antimicrobial Plastic Preparation

The procedure followed was based on the work of Bastarrachea and Goddard, and by Bastarrachea (Bastarrachea, 2019; Bastarrachea & Goddard, 2016). A mixture of 50% PP and 50% Polybond 7200 (w/w) was blended and extruded using a Laboratory Mixing Extruder equipped with 1/8 in orifice strand die (Dynisco, Franklin, MA, USA) at 180 °C and 20 rpm. The strands of this blend, referred to as PP-MA (Figure 3.1), were then cut into pellets using a LEC Chopper (Dynisco, Franklin, MA, USA). The pellets were hot pressed at 200 °C and 60 MPa. The thickness of the films was measured to be 0.28 ± 0.03 mm. The films were then cut into 2×2 cm coupons and cleaned in an ultrasonic bath as follows. The cleaning consisted of 2 cycles of acetone (10 min each) and 2 cycles of deionized (DI) water (10 min each). The coupons were then dried under anhydrous calcium sulfate overnight ($\sim 20\%$ relative humidity).

The 2×2 cm coupons were then spin coated with MVE and chitosan as follows. A solution of MVE in acetone was prepared (1 mg/mL) and sonicated for 10 min to completely dissolve the MVE. Chitosan solubilized in 1% acetic acid (15 mg/mL, pH value of 2.86 ± 0.02), was equally prepared by sonicating for 10 min. Then, 0.3 mL of the MVE solution was applied onto a 2×2 cm PP-MA coupon and spin-coated for 1 min at 2000 rpm (2000 rpm acceleration) with a VTC-200P spin coater (MTI Corporation, Richmond,

CA, USA). Afterwards, 0.3 mL of chitosan solution was applied and spin-coated over the MVE layer under the same conditions. The same procedure was repeated on the other side of the PP-MA coupon. The coupons were then dried under anhydrous calcium sulfate for 30 min and then heat cured for 1 h at 185 °C. The curing step was used to create covalent bonds with anhydride groups in MVE and amine groups in chitosan to produce imide rings as seen in Figure 3.1 (Bastarrachea, 2019; Bastarrachea & Goddard, 2016). This antimicrobial plastic was referred to as PP-MVE-CHI thereafter.

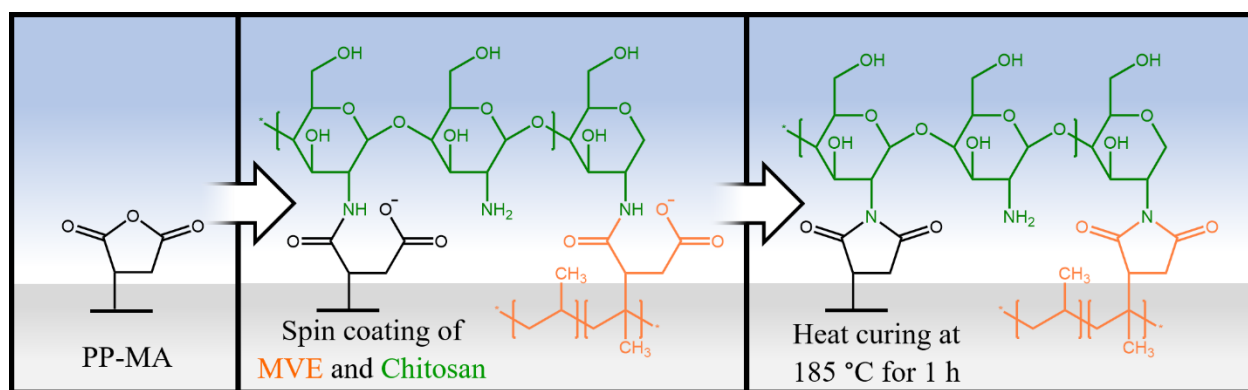


Figure 3.1 Steps in preparing PP-MVE-CHI.

Surface Characterization by Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy and Contact Angle Goniometry

The procedure outlined was also followed based on previous reports (Bastarrachea, 2019; Bastarrachea & Goddard, 2016). The surface chemistry at every step of the preparation of PP-MVE-CHI was analyzed through ATR-FTIR with an IRTracer-100 infrared spectrometer (Shimadzu Corporation, Japan) equipped with a diamond ATR crystal (Quest Single Reflection ATR Accessory, Specac Limited, United Kingdom) as follows. A coupon was placed on the diamond ATR crystal and at least 2 spots of the surface were analyzed from each side. The coupons were analyzed with Happ-Genzel appodization at a

4 cm⁻¹ resolution. A total of 32 scans were taken of each spot. After baseline correction was applied, characteristic bands were interpreted with the software KnowItAll (Biorad Laboratories, Philadelphia, PA, USA).

Contact angle goniometry was also done on the antimicrobial plastics prior to and following the reusability evaluation (as described later). A PP-MA coupon was also evaluated as a control. The hydrophobicity of the samples was measured with a VCA optima digital contact angle instrument (AST products, Billerica, MA, USA). A 1 µL droplet of DI water was deposited on the samples and the static contact angle (θ_s) was measured on at least 1 spot per surface. Images were captured with the instrument's software and results were analyzed from the tangent lines. At least 3 replicates per treatment were analyzed.

Test Microorganism

The test microorganism, Gram-negative bacterium *E. coli* K12 ATCC 47009 was kindly provided by Prof. Jeffrey Broadbent of the department of Nutrition, Dietetics, and Food Sciences at Utah State University (Logan, UT, USA). A loopful of the frozen stock culture (kept at – 80 °C) in 20% glycerol was taken, inoculated by streaking across a TSA plate, and incubated at 37 °C for 24 h. A single colony was then inoculated in TSB and incubated at 37 °C for 12 – 14 h overnight. A loopful of the overnight TSB was inoculated by streaking onto a new TSA plate and incubated at 37 °C for 24 h. These TSA plates were stored under refrigeration for up to 3 weeks for future antimicrobial evaluations. For antimicrobial and reusability evaluations (described below), a single colony was inoculated in 9 mL of sterile TSB and incubated at 37 °C overnight for 12 – 14 h

(Bastarrachea, 2019). This overnight culture in stationary phase would be used to prepare the bacterial suspensions for the evaluations explained in the following subsection.

Antimicrobial And Reusability Evaluation

The following procedure was based on the work done by Bastarrachea, and Bastarrachea and Goddard (Bastarrachea, 2019; Bastarrachea & Goddard, 2016). To determine the reusability and antimicrobial effectiveness, the as prepared 2×2 cm PP-MVE-CHI coupons were cut into 1×1 cm squares and challenged against *E. coli* K12. The aqueous bacterial suspension was prepared by diluting the overnight TSB 0.1% with sterile DI water to a final microbial load of $\sim 6 \log(\text{CFU/mL})$. The as prepared PP-MVE-CHI coupons were protonated in 0.1 N HCl (pH 1, prepared using sterile DI water and 1 N HCl) 3 times (2 mL of 0.1 N HCl per 1×1 cm coupon), leaving them in the last rinse for 20 min. Then, the coupons were allowed to dry under anhydrous calcium sulfate for 30 min. Once dry, six 1×1 cm coupons were collectively placed in contact with 1 mL of the bacterial suspension inside a capped glass test tube (13 mm diameter). The coupons were either exposed to 30 min of UV-A light ($\lambda = 365$ nm, $6.1 \pm 0.2 \mu\text{W}/\text{cm}^2$) inside a UV crosslinker CL-1000L (Analytik Jena, Jena, Germany) or kept in the dark at room temperature (25 °C). The vertical distance between the UV-A light bulbs and the bacterial suspensions inside the test tubes was 15 cm. As controls, 1 mL of the bacterial suspension was placed in a capped glass test tube (13 mm diameter) and exposed to UV-A light or left in the dark for 30 min. PP-MA coupons were also protonated and dried under the same conditions applied to PP-MVE-CHI and exposed to 1 mL of the bacterial suspension in a capped glass test tube and exposed to the same UV-A conditions for 30 min as another control. After the 30 min,

serial 10% dilutions of the bacterial suspensions from each treatment were prepared with 0.1% PW and 0.1 mL of each dilution was inoculated onto individual TSA plates in duplicate. To reduce the limit of detection to 1 log(CFU/mL), 1 mL of the first dilution was inoculated onto 3 TSA plates (333 μ L per plate). The plates were incubated at 37 °C for 48 h. The number of survivors were enumerated thereafter through plate counts.

The reusability evaluation was conducted as follows. After the procedure described, the PP-MVE-CHI coupons tested under UV-A light were again rinsed in 0.1 N HCl as explained, allowed to dry, and exposed again to fresh bacterial suspension newly prepared in the same way and under the same UV-A conditions. This protocol was repeated for a total of 10 cycles. As a control, 1 mL of bacterial suspension alone under the same UV-A light conditions was used (this treatment was used as a control to ensure the UV-A light source remained harmless to the bacteria across the 10 cycles). In each cycle, number of survivors were determined through plate counts as explained before. A total of 3 replicates were performed in these analyses, with 3 separately prepared sets of PP-MVE-CHI coupons.

Primary Amine Content Through Acid Orange 7 Analysis

The primary amine content of the as prepared PP-MVE-CHI, PP-MVE-CHI after 10 cycles of reusability evaluation, and PP-MA coupons (control) was analyzed with the colorimetric AO7 assay (Bastarrachea, 2019; Uchida, Uyama, & Ikada, 1993) as follows. Each 1 \times 1 cm coupon was individually immersed in test tubes with 5 mL of 1 mM AO7 aqueous solution at pH 3, adjusted with 1 N HCl, and left shaking for 3 h. Then, the coupons were rinsed in copious amounts of pH 3 water, three times to desorb any unbound AO7 dye. The

coupons were then individually placed in a test tube with 5 mL of pH 12 water (adjusted with concentrated sodium hydroxide) and left shaking for 15 min to desorb the bound AO7 dye. The absorbance of the solutions in contact with the coupons was then measured at 455 nm with a SpectraMax® iD3 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The AO7 concentration was determined from a previously prepared standard curve. The primary amine concentration was based on the surface of the PP-MVE-CHI coupons and the amount of dye absorbed (expressed in nmol/cm², 1 mol of AO7 equals 1 mol of primary amine). At least 3 replicates from each treatment was conducted with separately prepared PP-MVE-CHI coupons.

Surface Analysis by Atomic Force Microscopy

After the 10 cycles of reusability evaluation described above, a replicate from the PP-MVE-CHI coupons was taken and analyzed through AFM as follows. Images were taken on a Nanoscope III Bioscope (Digital Instruments, Inc., NY, USA) in tapping mode. Budget Sensors-Tap 300AL-G cantilevers with a tip radius of curvature < 10 nm, 125 µm length, 30 µm width, 4 µm thickness and a 40 N/m force constant were employed. Images were collected at 256 × 256 pixel resolution and 1 Hz over a range of scan sizes and angles.

Surface Analysis by Scanning Electron Microscopy

Following the 10 cycles of reusability evaluation as described above, the 1 × 1 cm PP-MVE-CHI coupons were taken and analyzed under SEM. For comparison, as prepared PP-MVE-CHI were also analyzed as follows. The PP-MVE-CHI coupons (either as prepared or subjected to the 10 cycles) were rinsed in copious amounts of sterile DI water to remove

any unbound bacteria and then immersed in absolute ethanol for 45 s (Djordjevic, Wiedmann, & Mcclandsborough, 2002). The coupons were then allowed to dry in anhydrous calcium sulfate for 30 min and sputter coated with gold and palladium with a rotary sputter coater system EMS150R ES (Electron Microscopy Sciences, Hatfield, PA, USA). The surfaces were analyzed with a FEI Quanta FEG 650 (Field Electron and Ion Company, Hillsboro, OR, USA) under low vacuum and at an accelerating voltage of 10 kV. Multiple images were taken across the surface of the coupons.

Fluorometric Evaluation of Reactive Oxygen Species Generation

This assay is based on the degradation of fluorescein when exposed to ROS (Bastarrachea, 2019; Nayak, Muniz, Sales, & Tikekar, 2016). The as prepared PP-MVE-CHI (six 1×1 cm) coupons were protonated in 0.1 N HCl, mimicking the same procedure described earlier. They were then exposed to the fluorometric probe fluorescein under the same UV-A conditions as described in the previous section to determine if PP-MVE-CHI generates ROS when exposed to UV-A light. The fluorescein solution was prepared by dissolving the fluorophore in DI water to achieve a 10 μ M solution. The coupons were then subjected to either the UV-A light conditions described for the antimicrobial evaluations or kept in the dark for 30 min at room temperature (25 °C). As controls, 1 mL of fluorescein probe alone either exposed to UV-A light or kept in the dark were used, as well as 1 mL of fluorescein solution in contact with six 1×1 cm² PP-MA coupons exposed to the same UV-A light conditions. At the end of the 30 min, 0.1 mL of the fluorometric probe was taken from each treatment and placed in individual wells of an opaque 96-well microplate. The fluorescence intensity was measured at an excitation/emission of 480 nm/515 nm, with

a SpectraMax® iD3 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Relative fluorescence values were obtained by calculating the natural logarithm of the ratio between the fluorescence intensity of the corresponding treatment and the fluorescence intensity of the fluorometric probe solution alone kept in the dark (Bastarrachea, 2019).

Cell Morphology Analysis by Scanning Electron Microscopy

To evaluate changes in cell morphology, the protocol reported by Kihm, et al. (Kihm, D. J., Leyer, G. J., An, G. H. & Johnson, 1994) and Wang et al. (Q. Wang, De Oliveira, Alborzi, Bastarrachea, & Tikekar, 2017) was applied. The antimicrobial evaluation under UV-A light was performed with as prepared PP-MVE-CHI coupons as explained before. A total of 5 test tubes each with 1 mL of *E. coli* K12 ($\sim 6 \log(\text{CFU/mL})$) and each with six $1 \times 1 \text{ cm}$ PP-MVE-CHI coupons were subjected to the 30 min of UV-A light exposure. As control, five test tubes each with the same volume of bacterial suspension alone were placed in the dark at room temperature for 30 min. The bacterial suspensions were aseptically recovered and filtered using a polycarbonate $0.2 \mu\text{m}$ sterile filter membrane (13 mm diameter, Merck Millipore Ltd., Tulagreen, Ireland). Then, the filters were immersed in 0.25% glutaraldehyde for 1 h, which was followed by rinsing in DI water for 3 times and dipping in increasing concentrations of ethanol in DI water: 10, 20, 50, 75, 90 and 100% (v/v). The filters were then allowed to dry under anhydrous calcium sulfate. Before SEM analysis, the filters were sputter coated with gold and palladium with a rotary sputter coater system EMS150R ES (Electron Microscopy Sciences, Hatfield, PA, USA). The surfaces were analyzed with a FEI Quanta FEG 650 (Field Electron and Ion Company,

Hillsboro, OR, USA) under low vacuum and at an accelerating voltage of 15 kV. Multiple images were taken of each sample.

Statistical Analysis

Statistical analysis was performed to determine significant differences between treatments as appropriate. This was done by one-way Analysis of Variance (ANOVA) followed by Tukey's test with a confidence interval of 95% using the software Minitab® version 18 (Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

Surface Characterization By Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy

Figure 3.2 depicts the infrared spectra at every step of the antimicrobial plastic preparation. The anhydride group was confirmed in PP-MA with the presence of C=O vibration band at 1780 cm^{-1} and carboxylic acids at 1720 cm^{-1} . Once the MVE was spin-coated, the maleic anhydride groups could be found at 1850 cm^{-1} . The C–O–C vibration band, also from MVE, can be found in the $1175 - 1038\text{ cm}^{-1}$ range. After spin-coating chitosan, the C–N vibration band of primary amines was found between in the $1090 - 1068\text{ cm}^{-1}$. Polymeric –OH vibration from chitosan was found in the $3400 - 3200\text{ cm}^{-1}$ range. The presence of amides was confirmed from the bands that belong to their C=O and N–H vibrations at $1680 - 1630\text{ cm}^{-1}$ and $1570 - 1515\text{ cm}^{-1}$, respectively, which confirms initial crosslinking between MVE and chitosan. After heat curing at $185\text{ }^{\circ}\text{C}$, it is expected that imide rings form, which could be confirmed by the C=O vibration band at $1755 - 1680\text{ cm}^{-1}$. It is

presumed that the number of primary amines and amides decrease due to cross-linking after heat curing, which can be confirmed by the decrease in absorbance of the bands at $1090 - 1068 \text{ cm}^{-1}$ and $1680 - 1630 \text{ cm}^{-1}$. It is also assumed that the $-\text{OH}$ groups of chitosan decrease due to formation of ester bonds as they cross-link with the anhydride groups of MVE (Smith, 1999).

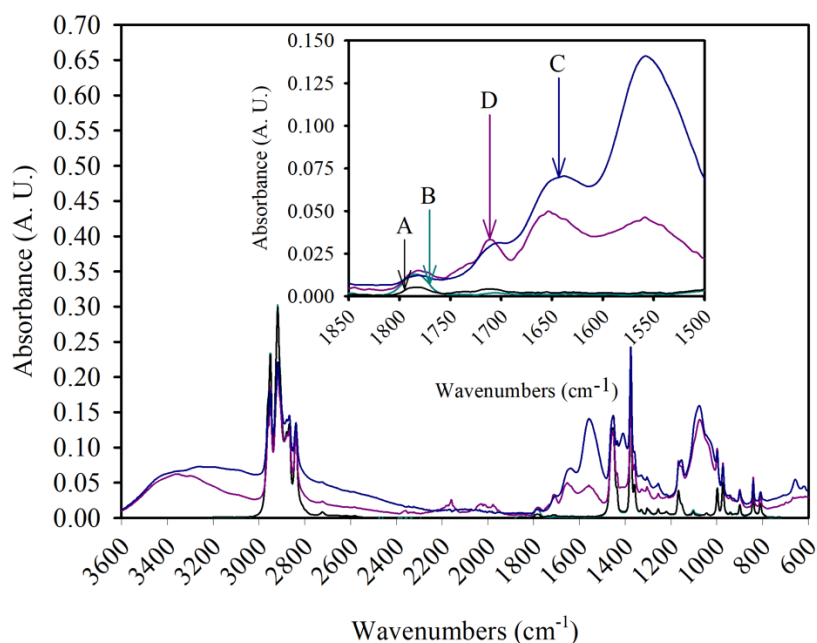


Figure 3.2 Infrared spectra of PP-MA (A), spin-coated MVE (B), spin-coated chitosan (C) and finished PP-MVE-CHI after heat curing at 185°C (D).

Antimicrobial and Reusability Evaluation

When challenged against *E. coli* K12 under UV-A light, PP-MVE-CHI was able to significantly ($P < 0.05$) reduce the microbial population as compared to controls (Figure 3.3). PP-MVE-CHI reduced the microbial load by $95.9 \pm 0.2\%$ with respect to the bacterial suspension alone kept in the dark and by $95.3 \pm 0.7\%$ with respect to the bacterial suspension alone exposed to UV-A light. As expected, PP-MA did not show a significant

antimicrobial effect ($P > 0.05$). When kept in the dark, PP-MVE-CHI did provide a significant ($P < 0.05$) reduction in the microbial load as compared to the bacterial suspension kept in the dark ($29.7 \pm 11\%$), but that effect was significantly ($P < 0.05$) lower than the one observed under UV-A light, where more than 1 logarithmic reduction (more than 90% reduction) was reached. As expected, this difference may be brought by UV-A light and the resulting ROS generation from the cationic material (Bastarrachea, 2019). Skowron et al. were able to find similar results in an antimicrobial experiment against *L. monocytogenes*, in which they isolated *L. monocytogenes* from various food sources and placed them in contact with chitosan films. They found between 1.2 – 1.9 log(CFU/cm²) reduction when placed in contact with the films directly after preparation and 3.5 – 4.0 log(CFU/cm²) reduction when placed in contact with the films stored for 10 days. The films were in contact with the bacteria for 0, 1, 6, 12, and 24 h (Skowron et al., 2019). Vilela et al. were also able to show antimicrobial effects of chitosan in conjunction with ellagic acid when placed in contact with Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* for 24 h. They observed ~ 6 log(CFU/mL) reduction with both strains regardless of the chitosan/ellagic acid mixture (Vilela et al., 2017). It should be noted, however, both of these studies conducted their antimicrobial evaluations for longer periods of exposure time.

Figure 3.3 also shows the results of the reusability evaluation. PP-MVE-CHI demonstrated to be reusable across the 10 cycles, with no evidence of decay in antimicrobial efficacy. The microbial reduction as compared to the control was $94.0 \pm 3.3\%$ throughout the 10 cycles.

The final section of Figure 3.3 displays the IR spectra of the as prepared PP-MVE-CHI and the PP-MVE-CHI after the 10 cycles of reusability evaluation. As can be noted, there is a slight increase in the presence of amides as confirmed from $1680 - 1630\text{ cm}^{-1}$ and $1570 - 1515\text{ cm}^{-1}$. This could be attributed to further crosslinking between the anhydride rings of MVE and the primary amines of chitosan, as well as imide-ring opening.

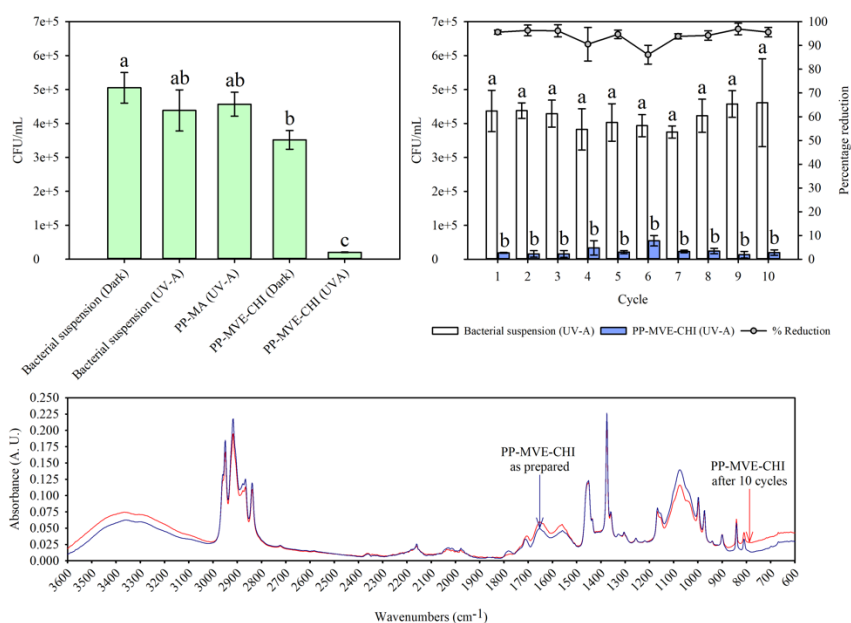


Figure 3.3 Top left: antimicrobial evaluation from all the treatments tested. Top-right: reusability evaluation. Bottom: IR spectra of PP-MVE-CHI before and after the 10 cycles of reusability evaluations. In each graph, treatments that share the same lowercase letter are not significantly different ($P > 0.05$).

Figure 3.4 (top) shows that the hydrophobicity decreased after the 10 cycles of reusability evaluation. It was found that there was a significant ($P < 0.05$) effect as compared to the as prepared PP-MVE-CHI and PP-MA. Ye et al. (Ye, Chen, Zhang, Zhang, & Shen, 2014). Conducted a study where they modified a silicon plate by layer-by-layer

electro-assembly of chitosan and lignosulfonate. They found that at a voltage of 48 V, the water contact angle was 78.9° . This is similar to what was found with PP-MVE-CHI.

The as prepared PP-MVE-CHI exhibited an almost hydrophobic character, with a θ_s value of $87.6 \pm 0.5^\circ$, significantly ($P < 0.05$) lower than the value given by PP-MA, $104.8 \pm 2.8^\circ$ (Figure 3.4, top). After the 10 cycles of reusability evaluation, the θ_s value of PP-MVE-CHI significantly ($P < 0.05$) reduced to $79.0 \pm 3.0^\circ$. As explained earlier in this subsection, this may have been a result of a continued anhydride rings opening throughout the cycles of reusability.

After conducting the AO7 assay, a slight decrease in primary amines on the surface of PP-MVE-CHI was observed after treating the material to 10 cycles of reusability evaluation, as shown in Figure 3.4 (bottom), going from 52.9 ± 14.2 to 48.7 ± 4.7 nmol/cm². This decrease, however, was not significant ($P > 0.05$). This agrees with the marginal effect on the overall surface chemistry confirmed by the ATR-FTIR analyses (Figure 3.3, bottom).

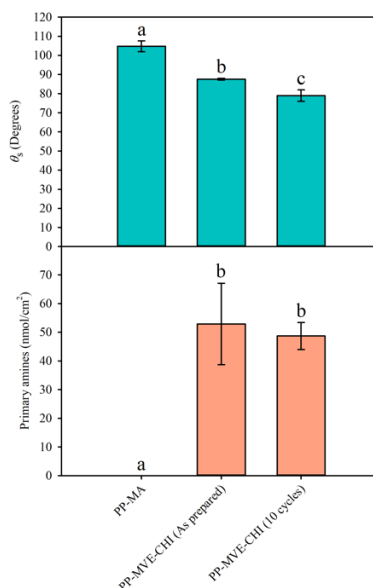


Figure 3.4 Contact angle goniometry (top) and AO7 assay results (bottom). Treatments that share the same lowercase letter are not significantly different ($P > 0.05$).

Surface Analysis by Scanning Electron Microscopy and Atomic Force Microscopy

SEM analyses of the surface of PP-MVE-CHI before and after the 10 cycles of reusability didn't show substantial changes in the material, and no evidence of fouling or cell attachment was observed (Figure 3.5). This is also in agreement with the IR spectra shown in Figure 3.3. In addition, this finding confirms the stability of PP-MVE-CHI, which results in its reusability.

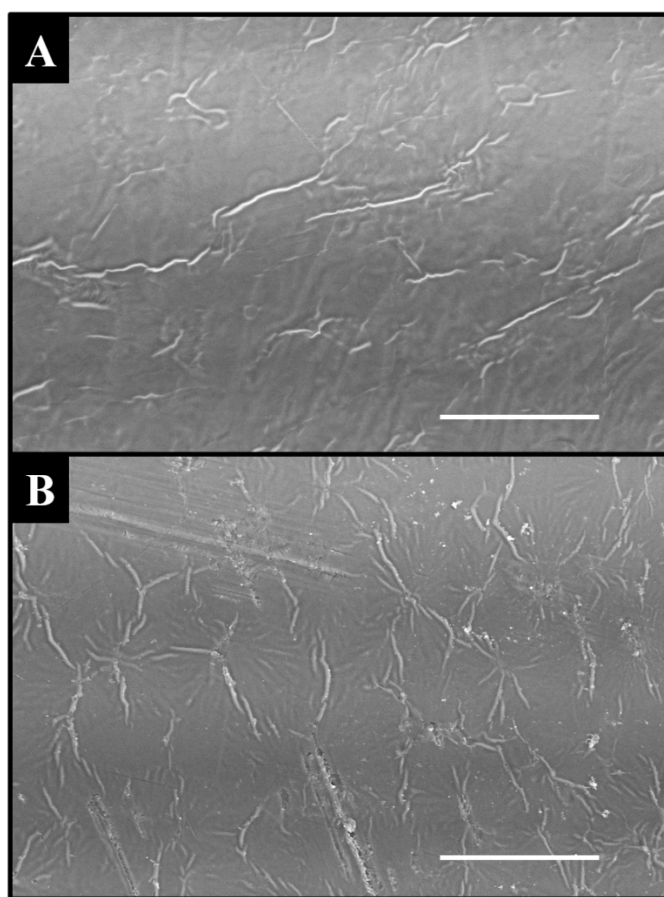


Figure 3.5 SEM images of the surface of as prepared PP-MVE-CHI (A) and PP-MVE-CHI after 10 cycles of reusability (B). Scale bar represents 50 μm .

Figure 3.6 shows the AFM pictures obtained. The PP-MA (A) surface is similar to what has been found in previous work (Bastarrachea & Goddard, 2016). The as prepared

PP-MVE-CHI (B) confirmed the slightly organized pattern resembling “hills”. After the 10 cycles of reusability (C and D), no evidence of cell fouling was observed. However, the roughness seemed to have decreased as compared to the as prepared PP-MVE-CHI. A possible reason behind this could be further crosslinking between the amine groups of chitosan and the anhydride groups of MVE, as suggested by the AO7 analysis (Figure 3.4).

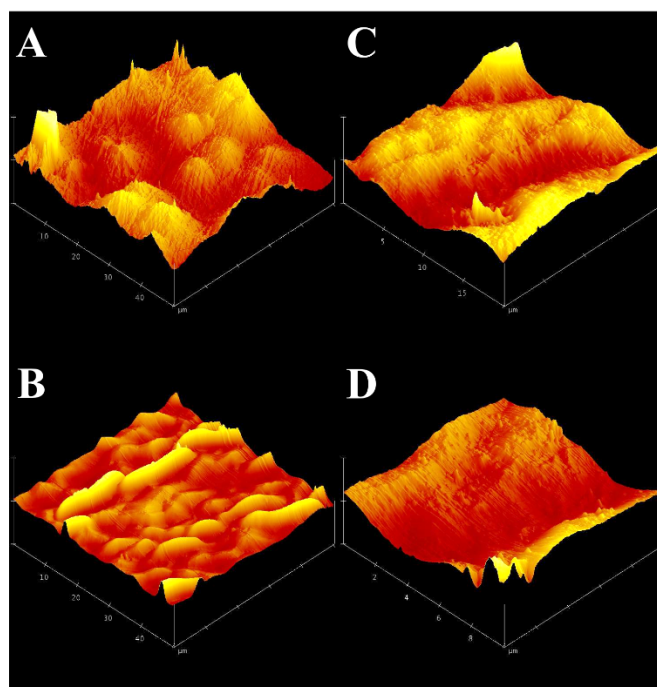


Figure 3.6 AFM images of PP-MA (A, vertical axis interval: 500 nm), as prepared PP-MVE-CHI (B, vertical axis interval: 2 μ m), and PP-MVE-CHI after 10 cycles of reusability (C, vertical axis interval: 500 nm; D, vertical axis interval: 300 nm).

Fluorometric Evaluation of Reactive Oxygen Species

As shown in Figure 3.7, a significant effect ($P < 0.05$) was observed in the value of relative fluorescence from both the PP-MVE-CHI kept in the dark (-4.1 ± 0.1) and under UV-A light (-5.1 ± 0.2), which confirms that PP-MVE-CHI is able to generate ROS upon UV-A light exposure at a significantly higher proportion than when kept in the dark. As expected, UV-A light alone under the conditions tested didn't seem to promote ROS generation, from

the marginal value of relative fluorescence given by the fluorescein solution alone (0.0 ± 0.1). These results are supported by previous works that indicate that polycations are able to generate ROS when exposed to certain wavelengths of light and even in the dark (Y. Wang et al., 2013).

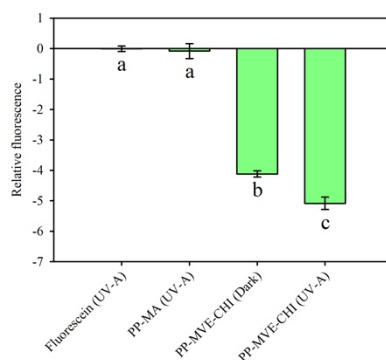


Figure 3.7 Fluorometric evaluation of ROS generation. Treatments that share the same lowercase letter are not significantly different ($P > 0.05$).

Cell Morphology Analysis By Scanning Electron Microscopy

An observable change in cell morphology was found from the exposure to PP-MVE-CHI and UV-A light as compared to the control *E. coli* K12 cells (Figure 3.8). The controls cells exhibited the usual bacillar shape of *E. coli*. In contrast, the treated cells exhibited a rough, irregular, and altered membrane, and overall structural damage. This may be a result of the cationic nature of PP-MVE-CHI and the generation of ROS, which are able to disrupt and destabilize the cell membrane and other cell components (Bastarrachea, Denis-Rohr, et al., 2015; Verlee et al., 2017).

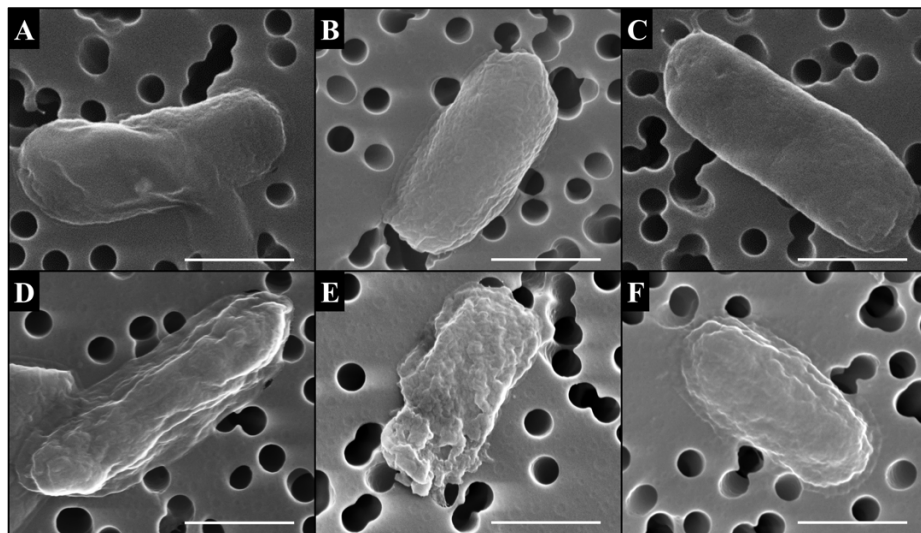


Figure 3.8 Cell morphology of control *E. coli* K12 cells (A-C) and cells exposed to PP-MVE-CHI under UV-A light (D-F). Scale bars represent 1 μm .

CONCLUSION

This study has shown that the PP-MVE-CHI plastic is a robust and effective antimicrobial material. The use of UV light may have also assisted in reducing the microbial load through the generation of ROS. Since both chitosan and methyl vinyl ether have low toxicity to humans, it is safe for them to be used in food packaging (Jones, Lavery, Morris, & Andrews, 2016; Kong et al., 2010). The main drawback to chitosan is that it is not very soluble (Sun et al., 2017).

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CHAPTER 4

ZEIN-MODIFIED ANTIMICROBIAL POLYPROPYLENE: CHARACTERIZATION AND REUSABILITY UPON UV-A LIGHT EXPOSURE²

ABSTRACT

Zein is a protein found in the endosperm of corn kernels. A variety of food-related applications have been explored with it, due to its multiple functionalities. However, it doesn't have intrinsic antimicrobial properties. In this study, polypropylene was modified through reactive blending for it to be able to harbor zein. The modified material showed to be antimicrobial against *E. coli* K12 under UV-A light exposure. Fluorometric techniques suggested that this mechanism is driven by generation of reactive oxygen species. In addition, the zein-modified polypropylene was able to be reused for 10 cycles without detrimental effects, providing ~ 90% reduction in the microbial population across the 10 cycles. Its stability was confirmed through spectroscopic and microscopic techniques, that showed marginal effects on its chemical and physical integrity. In addition, electron microscopy showed visible damage in the morphology of the cells exposed to it.

² LWT- Food Science and Technology. Zein-Modified Antimicrobial Polypropylene: Characterization and Reusability Upon UV-A Light Exposure.² Volume 121, March 2020, 108983. Andrew T. Gagon, David W. Britt, Luis J. Bastarrachea. (original copyright notice as given in the publication in which the material was originally published) ".

INTRODUCTION

According to the Center for Disease Control (USA), there have been 25,606 infections reported due to food-borne pathogens, which represents an increase of 12% in the past three years (Tack et al., 2019). The food industry, among others, has employed physical and chemical sanitization, such as heat and disinfectants, to reduce the amount of contamination on processing equipment and surfaces. However, the efficacy of these techniques may be reduced by microbial resistance or fouling due to organic matter and biomass (Bastarrachea & Goddard, 2016).

In addition to the application of disinfectants in processing facilities, preservation methods such as heating and cooling are also necessary to limit or eliminate microbial load in foods. These preservation methods include sterilization (canning), pasteurization, drying, freezing, acidification, water activity control, modified atmosphere packaging, smoking, and the use of chemical preservatives (Floros et al., 2010). Each of these methods is effective and has the ability to limit microbial growth, but consumers are beginning to look for a more natural or minimally processed means of preserving food (Mustapha & Lee, 2017). The traditional methods may also lead to losses of sensory quality and loss of nutrients (Sharif et al., 2017). This has led to the exploration of alternative methods of food preservation. One of these methods consists in the development of antimicrobial materials (Bastarrachea, Denis-Rohr, et al., 2015; Lucera, Costa, Conte, & Del Nobile, 2012). Using coatings with antimicrobial characteristics on machinery and materials found throughout the processing, handling, and storage of foods may be an alternative way to reduce microorganism growth or cross-contamination. Methods to prepare antimicrobial materials may include embedding the antimicrobial compound onto a surface, immobilizing the

agent on the surface (through electrostatic or covalent bonds), and polymer grafting (Bastarrachea, Wong, et al., 2015). Some compounds that have been used for antimicrobial coatings on different materials include nisin, essential oils, organic acids, peptides, triclosan, silver, quaternary ammonium salts, and TiO₂ (Bastarrachea, Wong, et al., 2015; Hoffman et al., 2016). Light-activated compounds are a group of compounds that achieve antimicrobial efficacy when exposed to a particular wavelength of light, which generate reactive oxygen species (ROS). The ROS are in turn able to react with biomolecules, such as nucleic acids and cell membranes, causing cell death (Bastarrachea, Denis-Rohr, et al., 2015). This category of antimicrobials have been used widely to prepare antimicrobial materials. In a recent study, a polyamide material was modified with the photoactive compounds Reactive Blue P-3R and 3,3',4,4'-Benzophenone tetracarboxylic acid, and exposed it to different wavelengths of light. They found that the 3,3',4,4'-Benzophenone tetracarboxylic acid was able to generate ROS. They also found that the polyamide modified with both compounds was able to reduce the amount of growth when challenged against *Escherichia coli* (between 23 and 99%, depending on the concentration of benzophenone) and *Staphylococcus aureus* (between 29 and 99% depending on the concentration of benzophenone) (Gao et al., 2017). In another study, TiO₂ was mixed with *E. coli* PHL1273 into a slurry and exposed to UV-A, UV-B, or UV-C. It was found that TiO₂ with UV-A was able to reduce to the growth $\sim 10^3$ CFU/mL (Benabbou et al., 2007). As mentioned earlier, proteins and peptides can also be used to modify materials and render them antimicrobial (Bastarrachea, Denis-Rohr, et al., 2015). Their antimicrobial mechanism works in a similar fashion as polycationic structures, in that they are able to disrupt the cell membrane integrity (through interaction with the negative charge of the

membranes), and inhibit proteins and DNA or RNA synthesis (Bahar & Ren, 2013). In a recent study, the peptides nisin and magainin were attached to a stainless steel surface and challenged against *Listeria ivanovii*, which resulted in 80 % and 65 % microbial reduction, respectively (Héquet, Humblot, Berjeaud, & Pradier, 2011). Another method to render a material antimicrobial is the use of polycations. Polycations are compounds that bear a positive charge, which causes electrostatic interactions with the cell membrane and leads to loss of fluidity in the membrane (Deka et al., 2015). However, the antimicrobial characteristics of polycations may be enhanced when exposed to light and they are also able to generate ROS (Ji, Corbitt, et al., 2011). Despite the possibilities antimicrobial materials may have as an alternative method, there are some disadvantages such as regulatory approval, loss and migration of active compounds, loss of efficacy, and lack of reusability due to low stability (Bastarrachea, Denis-Rohr, et al., 2015). One technique able to overcome the lack of stability of modified materials is reactive blending. Reactive blending consists in the mixing of two polymers (that have reactive functional groups) through blending, which results in the formation of covalent bonds between the different components and the formation of a material with a property of interest (Parameswaranpillai, Thomas, & Grohens, 2015). In a recent study, polypropylene was modified for it to harbor reactive anhydride rings and then blended with polyethyleneimine to form covalent imide bonds. The material was challenged against *E. coli* O157:H7, in the presence of horse serum as a simulant of organic matter. Depending on the organic matter concentration and the content of *N*-halamines, the material was able to provide more than 4 logarithmic reductions. The study also found that the material, after being exposed to

extreme pH levels, exhibited self-healing capabilities upon re-heating (Bastarrachea & Goddard, 2016).

Zein is a ~35 KDa protein found in the endosperm of corn kernels. It has been studied in applications that involve controlled release of compounds, as adhesives, biodegradable materials, and edible films (Rishi & Munir, 2001). However, zein is not inherently antimicrobial, which may be why much of the work that has been done with zein has involved imbedding it with antimicrobial compounds in controlled release applications (Hoffman et al., 2016; Li, Guo, & Heinamaki, 2010). In a study by Li et al., tablets containing metoprolol tartrate were coated with a zein film. It was found that the zein coated tablets released at a controlled rate, while the tablet alone dissolved quickly (Li et al., 2010). Zein has been a compound of interest for these type of applications due to its natural origin and due to its hydrophobicity and alcohol solubility, which may provide better barrier properties against the release of certain compounds (Tihminlioglu et al., 2010). Zein also has an isoelectric point of 6.8, which implies that below a pH of 6.8 it will harbor a positive charge (Bouman et al., 2016). We hypothesize that if positively charged, zein would be able to inactivate microorganisms upon UV light exposure.

In the present study, polypropylene was modified for it to harbor reactive anhydride groups and be able to react with the protein zein through covalent bond formation, and form a stable antimicrobial material through reactive blending. The use of UV-A light was explored as a means to enhance or trigger its antimicrobial effect. It was hypothesized that by protonating zein and exposing it to UV-A light, it would be possible to promote generation of reactive oxygen species and provoke microbial inactivation. The material was challenged against *Escherichia coli* K12 for 10 cycles to test its antimicrobial efficacy

and reusability. This was complemented with microscopic and spectroscopic techniques to test its stability towards repeated use. Possible antimicrobial mechanism and effects on cell structure were also explored.

MATERIALS AND METHODS

Materials

Isotactic polypropylene (PP) was from Scientific Polymer Products (Ontario, NY, USA). Polybond 7200, a maleic anhydride-grafted polypropylene, was graciously provided by Dr. John Yun from SI group, Inc. (Schenectady, NY, USA). Zein was from TCI America (Portland, OR, USA). Acetone, absolute ethanol, sodium hydroxide, acetic acid (95%), glutaraldehyde (25%) and hydrochloric acid (1 N) were from Fisher Scientific (Pittsburgh, PA, USA). Anhydrous calcium sulfate was from Drierite (W. A. Hammond DRIERITE Co. LTD, Xenia, OH, USA). The dye Acid Orange 7 (AO7) was from Electron Microscopy Sciences (Hatfield, PA, USA). Fluorescein disodium salt (376.27 g/mol) was from Acros Organics (Fair Lawn, NJ, USA). Tryptic soy agar (TSA) and tryptic soy broth (TSB) were from Difco, Becton Dickinson (Sparks, MD, USA). Peptone water (PW) was from Oxoid (Thermo Fisher Scientific, Waltham, MA, USA).

Antimicrobial Plastic Preparation

The methods below are based on the procedures outlined previously (Bastarrachea, 2019; Bastarrachea & Goddard, 2016). A 1:1 mixture (weight basis) of PP and Polybond 7200 was blended and extruded using a Laboratory Mixing Extruder equipped with a strand die with a 1/8 in orifice (Dynisco, Franklin, MA, USA) at 180 °C and 20 rpm. The mixture,

referred as PP-MA hereafter, was cooled and chopped into pellets using a LEC Chopper (Dynisco, Franklin, MA, USA). The pellets were hot pressed into films at 200 °C and 60 MPa. The thickness of the films was measured to be 0.28 ± 0.03 mm. The PP-MA films were cut into 2×2 cm coupons and cleaned in an ultrasonic bath consisting of 2 cycles of fresh acetone and 2 cycles of fresh deionized (DI) water (10 minutes per cycle). The cleaned PP-MA coupons were then allowed to dry under anhydrous calcium sulfate overnight ($\sim 20\%$ relative humidity).

Once dry, the 2×2 cm coupons were spin coated with zein as follows. A solution of zein was solubilized in 90% ethanol in DI water (0.05 g/mL) and sonicated for 10 min to completely dissolve the zein. Then, 0.3 mL of the zein solution was spin-coated for 1 min at 2000 rpm (2000 rpm acceleration) with a VTC-200P spin coater (MTI Corporation, Richmond, CA, USA). The other side of the coupon was equally coated in the same manner. The coupons were heat cured at 185 °C for 1 h. These coupons were referred to as PP-Z (Figure 4.1).

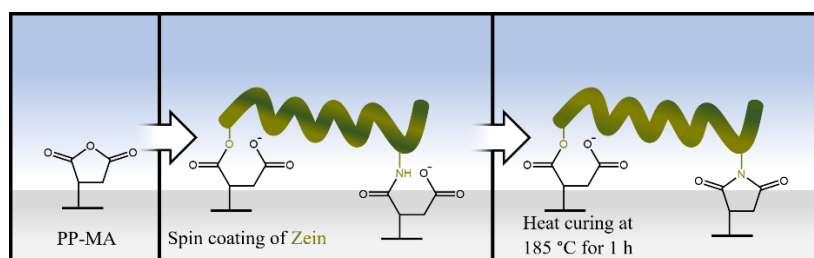


Figure 4.1 Depiction of the antimicrobial plastic preparation.

Surface Characterization By Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy And Contact Angle Goniometry

This procedure was based on work outlined previously (Bastarrachea, 2019; Bastarrachea & Goddard, 2016). The surface chemistry of the PP-Z coupons was analyzed at every step

of their preparation through Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) with an IRTracer-100 infrared spectrometer (Shimadzu Corporation, Japan) equipped with a diamond ATR crystal (Quest Single Reflection ATR Accessory, Specac Limited, United Kingdom) as follows. Coupons from independently prepared sets were randomly selected and placed on the diamond ATR crystal and multiple spots from the surface were analyzed using Happ-Genzel apodization at a 4 cm^{-1} resolution. A total of 32 scans were taken of each spot. After baseline correction was applied, characteristic bands were interpreted with the software KnowItAll (Biorad Laboratories, Philadelphia, PA, USA).

Contact angle goniometry was also conducted on the PP-Z coupons before and after the reusability evaluations (outlined in further subsection) as follows. A $1\text{ }\mu\text{L}$ droplet of DI water was placed on the samples and the static contact angle (θ_s) was measured with a VCA optima digital contact angle instrument (AST products, Billerica, MA, USA). At least 1 droplet per surface was applied for measurements and at least 3 replicates per treatment were performed.

Test Microorganism

The test microorganism, Gram-negative bacterium *E. coli* K12 ATCC47009, was graciously provided by Prof. Jeffrey Broadbent from the department of Nutrition, Dietetics, and Food Sciences at Utah State University (Logan, UT, USA). The frozen stock culture was kept in 20% glycerol at $-80\text{ }^{\circ}\text{C}$. A loopful was taken from the thawed stock, streaked over a TSA plate, and incubated at $37\text{ }^{\circ}\text{C}$ for 24 h. A colony from this plate was inoculated in TSB and incubated at $37\text{ }^{\circ}\text{C}$ for 12 – 14 h. A loopful of this TSB was then inoculated

onto a new TSA plate by streaking and incubated at 37 °C for 24 h. These plates were stored at 4 °C for up to 3 weeks for further evaluations. When needed, a single colony was inoculated in TSB and incubated at 37 °C for 12 – 14 h (Bastarrachea, 2019) to prepare the bacterial suspensions used in the evaluations described in the following subsections.

Antimicrobial Evaluation and Reusability

Before conducting the reusability evaluation, the antimicrobial efficacy of PP-Z and controls against *E. coli* K12 (with or without UV-A light exposure) was determined as follows. The as prepared PP-Z was cut into 1 × 1 cm coupons and rinsed in 0.1 N HCl (pH 1) three times (2 mL of 0.1 N HCl per 1 × 1 cm coupon) to promote their protonation, leaving the coupons immersed in the last rinsing solution for 20 min. Then, the coupons were allowed to dry for 30 min under anhydrous calcium sulfate. Once dry, six 1 × 1 cm PP-Z coupons were placed inside an individual sterile glass test tube (13 mm diameter) containing 1 mL of an aqueous suspension of *E. coli* K12 (~ 5 log(CFU/mL), prepared by making a 0.01% dilution of an overnight bacterial broth in sterile DI water). The test tube was capped and subjected to 30 min of UV-A light exposure ($\lambda = 365$ nm, 6053 ± 0.2 $\mu\text{W}/\text{cm}^2$) inside a UV crosslinker CL-1000L (Analytik Jena, Jena, Germany). The distance between the UV-A light source and the bacterial suspension was 15 cm (from the top of the chamber to the surface of the bacterial suspension). After the exposure time, serial 10% dilutions of the aqueous suspension were made in PW and 100 μL of each dilution were inoculated onto individual TSA plates. The plates were incubated at 37 °C for 48 h and the number of survivors determined through plate counts. The exact same procedure was performed in the dark to measure any antimicrobial effect from PP-Z without UV-A light

exposure. As an additional control, six 1×1 cm coupons of PP-MA (equally pre-protonated in 0.1 N HCl) were also immersed in 1 mL of bacterial suspension and subjected to the same UV-A light conditions. Bacterial suspensions alone (1 mL), either with UV-A light exposure or kept in the dark were also used as controls. These evaluations were performed 3 times (3 replicates), with independently prepared sets of PP-Z and PP-MA.

The reusability evaluation was performed as follows. After the first evaluation explained in the previous paragraph, the six PP-Z coupons exposed to UV-A light were recovered, rinsed again 3 times in 0.1 N HCl and immersed in the last rinsing solution for 20 min, allowed to dry, immersed in a new aqueous *E. coli* K12 suspension (~ 5 log(CFU/mL), 1 mL) and subjected to another 30 min cycle as described previously under the same UV-A light conditions. This process was repeated for a total of 10 cycles, and the microbial reduction in each cycle was determined through plate counts. The control was chosen to ensure the UV-A light source alone had no effect to the bacteria.

Primary Amine Content Determination Through Acid Orange 7 Analysis

Following the work of Uchida, Uyama, and Ikada (1993) and Bastarrachea (2019), an AO7 assay was done on the PP-Z coupons before and after the 10 cycles of reusability. A 1×1 cm PP-Z coupon from both before and after the 10 cycles and a PP-MA coupon was individually immersed in 5 mL of 1 mM AO7 aqueous solution (pH 3, adjusted with 1 N HCl) and allowed to shake for 3 h. After this period, the coupons were rinsed 3 times in copious amounts of pH 3 water to desorb any unbound dye. The coupons were then immersed in 5 mL of pH 12 water (adjusted with concentrated sodium hydroxide) and left to shake for 15 min to desorb any bound dye. The absorbance of each solution in contact

with the coupons was measured at 455 nm with a SpectraMax® iD3 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The AO7 concentration was determined from a previously prepared standard curve. The primary amine content was calculated based on the surface of the PP-Z coupons and the amount of dye absorbed (expressed in nmol/cm², 1 mol in solution of AO7 was equal to 1 mol of primary amine on the surface of PP-Z). At least 3 replicates from each treatment was evaluated.

Surface Analysis by Atomic Force Microscopy

Images of PP-Z coupons before and after the 10 cycles of reusability evaluation, as well as PP-MA coupons were captured through AFM as follows. Images were taken using a Nanoscope III Bioscope (Digital Instruments, Inc., NY, USA) in tapping mode. Budget Sensors-Tap 300AL-G cantilevers with a tip radius of curvature < 10 nm, 125 µm length, 30 µm width, 4 µm thickness and a 40 N/m force constant were employed. Images were collected at 256 × 256 pixel resolution and 1 Hz over a range of scan sizes and angles.

Surface Analysis by Scanning Electron Microscopy

SEM images were gathered of PP-Z coupons before and after the 10 cycles of reusability as follows. Following the 10 cycles, the coupons were individually rinsed in copious amounts of DI water to remove any unbound bacteria and then immersed in absolute ethanol for 45 s. The coupons were allowed to dry under anhydrous calcium sulfate for 30 min and then sputter coated with gold and palladium with a rotary sputter coater system EMS150R ES (Electron Microscopy Sciences, Hatfield, PA, USA). SEM pictures were obtained with a FEI Quanta FEG 650 (Field Electron and Ion Company, Hillsboro, OR,

USA) under low vacuum and at an accelerating voltage of 10 kV (Bastarrachea, 2019). Multiple images were captured from multiple spots in each treatment.

Fluorometric Evaluation of Reactive Oxygen Species Generation

A fluorometric assay based on the degradation of the fluorophore fluorescein upon ROS exposure was followed (Bastarrachea, 2019; Nayak et al., 2016). As prepared PP-Z coupons were cut into 1×1 cm coupons, pre-protonated in 0.1 N HCl, and allowed to dry as described in the antimicrobial section. Then, six 1×1 cm coupons were introduced in an individual glass test tube (13 mm diameter) containing 1 mL of a 10 μ M aqueous solution of fluorescein prepared with DI water. Then, the test tube was capped and subjected to 30 min of UV-A light exposure as described in the previous section, under the same conditions followed in the antimicrobial evaluations. As a control, six PP-Z 1×1 cm coupons were also put in contact with 1 mL of 10 μ M fluorescein for 30 min in the dark. As additional controls, 1 mL solutions of the same concentration of the fluorophore alone (without any material in them) were either exposed to the same UV-A light conditions or kept in the dark for 30 min. To rule out the generation of ROS by PP-MA, a last control consisted in exposing six 1×1 cm coupons of PP-MA to 1 mL of the same fluorophore solution under the same UV-A conditions for 30 min. After the 30 min, a volume of 100 μ L was taken from every treatment, introduced into individual wells of an opaque 96-well microplate, and the fluorescence was read at an excitation/emission of 480 nm/515 nm with a SpectraMax® iD3 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The values of fluorescence were recorded from each treatment, and the relative fluorescence was calculated by taking the natural logarithm of the ratio between the

fluorescence of the corresponding treatment and the fluorescence of the 10 μ M fluorescein solution kept in the dark for the 30 min. This evaluation was performed 3 times (3 replicates) with independently prepared sets of PP-Z coupons.

Cell Morphology Analysis by SEM

Changes in the morphology of *E. coli* K12 cells after exposure to PP-Z and UV-A light were evaluated as follows (Kihm, D. J., Leyer, G. J., An, G. H. & Johnson, 1994; Q. Wang et al., 2017). A total of 30 as prepared 1 \times 1 cm PP-Z coupons were protonated in 0.1 N HCl and allowed to dry as described in the antimicrobial evaluation section. Then, the PP-Z coupons were transferred into individual glass test tubes (13 mm diameter, 6 coupons per test tube). A volume of 1 mL of aqueous *E. coli* K12 ($\sim 6 \log(\text{CFU/mL})$, prepared as described in previous subsection) was introduced into each glass test tube. The 5 test tubes were then subjected to 30 min of UV-A light exposure following the same procedure explained in previous subsection. After the 30 min time, the bacterial suspension from the 5 test tubes was recovered and filtered through sterile polycarbonate 0.2 μ m filter membranes (13 mm diameter, Merck Millipore Ltd., Tulagreen, Ireland). The filter membranes were thereafter immersed in an aqueous solution of 0.25% glutaraldehyde for 1 h. Then, the membranes were rinsed 3 times in sterile DI water and dipped in aqueous solutions of increasing ethanol concentrations (10, 20, 50, 75, 90 and 100% v/v). Once exposed to the ethanol solutions, the filter membranes were allowed to dry under anhydrous calcium sulfate. Before SEM analysis, the filter membranes were sputter-coated with gold and palladium using an EMS150R ES device (Electron Microscopy Sciences, Hatfield, PA, USA) . SEM analysis was conducted with a FEI Quanta FEG 650 (Field Electron and Ion

Company, Hillsboro, OR, USA) under high vacuum and at an accelerating voltage of 15 kV. Multiple images of the surface were taken.

Statistical Analysis

Statistical analysis was done by determining significant differences between treatments where appropriate, using one-way Analysis of Variance (ANOVA) followed by Tukey's pairwise comparison using a 95% confidence interval with the software Minitab® version 18 (Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

Surface Characterization Through ATR-FTIR Spectroscopy

Figure 4.2 displays the infrared spectra during every step of antimicrobial plastic preparation. The anhydride group of PP-MA can be found at 1780 cm^{-1} . PP-MA also has a carboxylic acid vibration band that belongs to the C=O group at 1720 cm^{-1} . After spin-coating zein, a C=O vibration band from amides can be found at $1680 - 1642\text{ cm}^{-1}$. Amides were also confirmed with the N-H vibration band at $1570 - 1515\text{ cm}^{-1}$. After spin coating, the polymeric -OH group was also present in the $3400 - 3200\text{ cm}^{-1}$ range. After heat curing at $185\text{ }^{\circ}\text{C}$, the formation of imide rings was confirmed from their C=O vibration at $1755 - 1680\text{ cm}^{-1}$. Amides and amines are also confirmed from the C=O vibration band at $1750 - 1650\text{ cm}^{-1}$ and the C-N vibration band at $1320 - 1200\text{ cm}^{-1}$, respectively. The amides present after heat curing may be a result of cross-linking between PP-MA and the amine groups of zein.

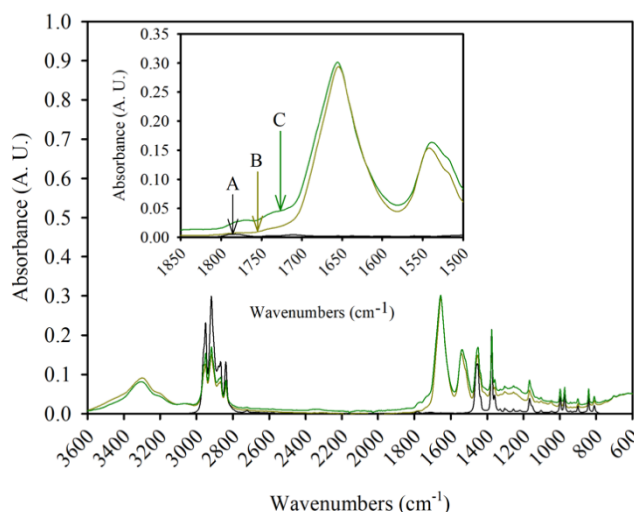


Figure 4.2 ATR-FTIR spectra of PP-MA (A), spin-coating of zein (B), and heat curing (C) to form PP-Z.

Antimicrobial Evaluations and Reusability

In combination with UV-A light exposure, PP-Z was able to significantly ($P < 0.05$) reduce the *E. coli* K12 population by $93.6 \pm 1.8\%$ with respect to the control bacterial suspensions kept in the dark, and by $92.6 \pm 2.6\%$ with respect to the control bacterial suspensions exposed to the same UV-A light conditions (Figure 4.3, top left). As expected, PP-MA did not show any significant ($P > 0.05$) antimicrobial effect. When kept in the dark, PP-Z was able to reduce the *E. coli* K12 population by $42.0 \pm 25\%$, but this reduction was not statistically significant ($P > 0.05$). A possible reason behind the apparent (although not significant) microbial reduction brought by PP-Z in the dark may be attributed to the ability that positively charged compounds have to produce ROS even in the dark (Ji, Corbitt, et al., 2011).

In combination with UV-A light, PP-Z was able to retain its antimicrobial efficacy (Figure 4.3, top right) across the 10 cycles of reusability, providing an $89.3 \pm 3.6\%$ reduction in the *E. coli* K12 population with respect to the control throughout the 10 cycles.

The infrared spectra of the surface of PP-Z was taken after the 10 cycles of reusability and compared with as prepared PP-Z coupons. The bottom image in Figure 4.3 shows that the surface chemistry varied only slightly when compared to an as prepared PP-Z coupon. A small decrease in imide rings was observed from the decrease in absorbance of the C=O band at $1755 - 1680\text{ cm}^{-1}$, which may be attributed from the opening of the imide rings. This can be supported from the increase in absorbance of the -OH groups at $3400 - 3200\text{ cm}^{-1}$, which may belong to the resulting carboxylic acids that appear after the imide rings are broken. Finally, there is a slight reduction in the presence of amines from the reduction in the C–N band at $1320 - 1200\text{ cm}^{-1}$. This may be explained from possible further cross-linking between the anhydride groups of PP-MA and the amine groups of zein throughout the 10 cycles.

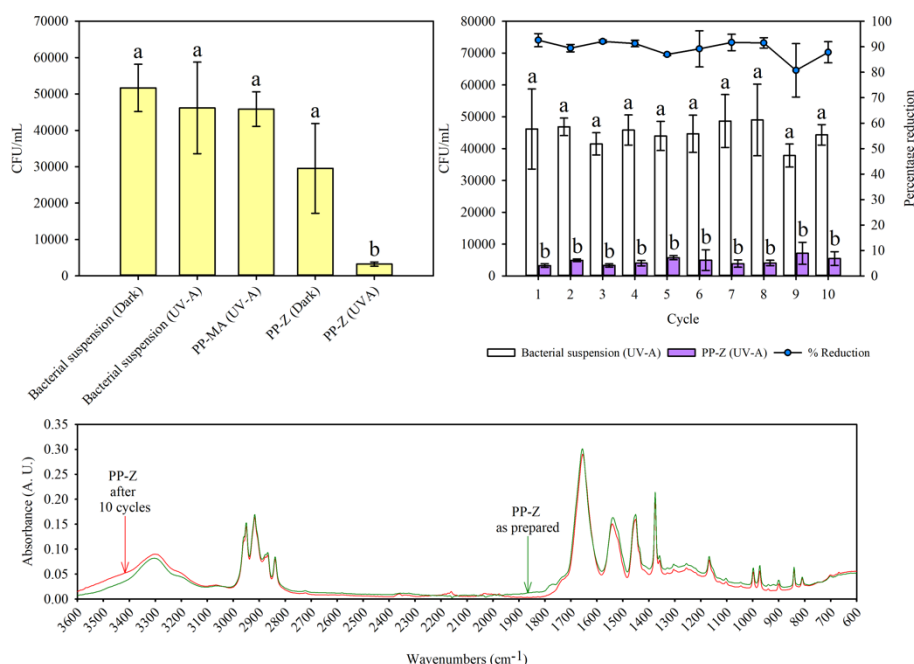


Figure 4.3 Top left: antimicrobial evaluation from all the treatments tested. Top-right: reusability evaluation. Bottom: IR spectra of PP-Z before and after the 10 cycles of reusability evaluations. In each graph, treatments that share the same lowercase letter are not significantly different ($P > 0.05$).

The as prepared PP-Z exhibited a hydrophobic character, with a θ_s value of $105.2 \pm 2.1^\circ$, which was not significantly different ($P > 0.05$) from the θ_s value given by PP-MA, $104.8 \pm 2.8^\circ$ (Figure 4.4, top). However, after the 10 cycles of reusability, PP-Z exhibited a significant ($P < 0.05$) reduction in its θ_s value, $91.9 \pm 2.9^\circ$, which (as explained before in this subsection) may be explained from the continued anhydride ring opening of PP-MA through the 10 cycles.

The bottom section of Figure 4.4 shows the primary amine content of PP-MA, as prepared PP-Z, and PP-Z after 10 cycles. As expected, PP-MA did not have any primary amines on its surface. There was also a significant difference ($P < 0.05$) between the as prepared PP-Z and the PP-Z after the 10 cycles of reusability, going from 8.3 ± 0.5 to $6.5 \pm 0.5 \text{ nmol/cm}^2$. This decrease may be attributed to the reduction in amines as cross-linking between zein and the anhydride groups of PP-MA may be happening throughout the 10 cycles.

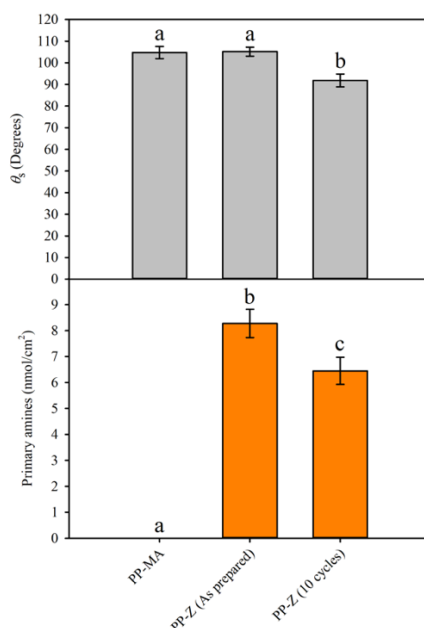


Figure 4.4 Contact angle goniometry (top) and AO7 assay results (bottom). Treatments that share the same lowercase letter are not significantly different ($P > 0.05$).

Surface Analysis by SEM and AFM

SEM images of as prepared PP-Z and PP-Z after the 10 cycles of reusability can be seen in Figure 4.5. The as prepared PP-Z exhibited a porous surface, which seemed to have flattened after the 10 cycles of reusability, preserving the apparent agglomerates found in the as prepared PP-Z. In a zein-related work, the protein was dissolved in 70% ethanol and then water mixed with a plasticizer and coated onto a tablet containing metoprolol tartrate. It was found that this type of coating had a similarly flattened surface as compared to a tablet coated with zein solubilized in 70% ethanol (Li et al., 2010). No evidence of bacterial attachment or biofouling was observed after the 10 cycles. This may be attributed to the hydrophobic character of PP-Z, which was preserved even after the 10 cycles.

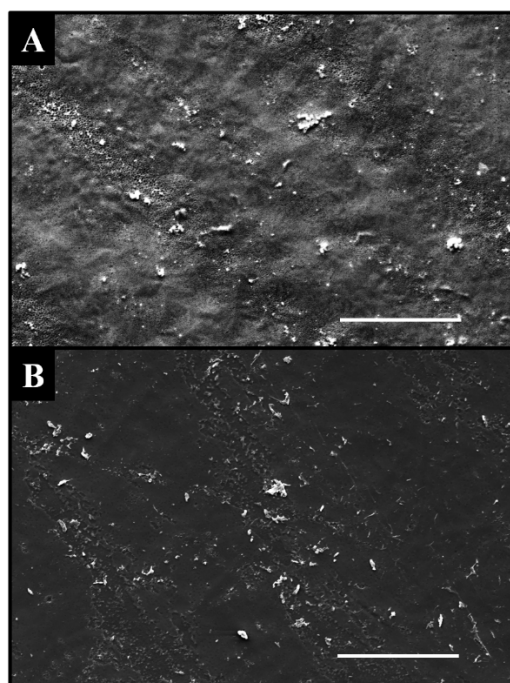


Figure 4.5 SEM analysis of the surface of PP-Z before (A, as prepared) and after (B) the 10 cycles of reusability. Scale bar represents 50 μm

Figure 4.6 displays the AFM images captured as follows: PP-MA (A), as prepared PP-Z (B and C), and PP-Z after the 10 cycles of reusability (D-F). As prepared PP-Z exhibited a porous surface, which may have contributed to its hydrophobic character (Luís, Domingues, & Ramos, 2019). After the 10 cycles of reusability evaluation, that porous pattern seemed to have been replaced by a more irregular and rougher surface. As it was also confirmed through SEM (Figure 4.5), the AFM results revealed no evidence of cell attachment after the 10 cycles. Similar porous images were captured by Ghanbarzadeha and Oromiehi while working with a zein film mixed with olive oil (Ghanbarzadeh & Oromiehi, 2008).

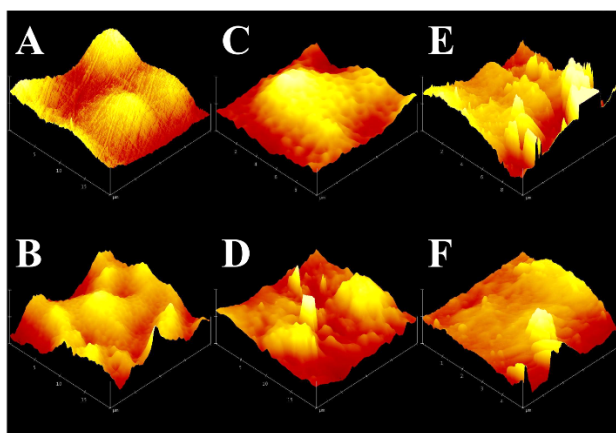


Figure 4.6 AFM images of PP-MA (A, vertical axis interval: 300 nm), as prepared PP-Z (B, vertical axis interval: 300 nm; C, vertical axis interval: 200 nm), and PP-Z after 10 cycles of reusability (D, vertical axis interval: 500 nm; E, vertical axis interval: 300 nm; F, vertical axis interval: 300 nm).

Fluorometric Evaluation of ROS

The assay followed was based on the fact that fluorescein degrades when it is exposed to ROS (Bastarrachea, 2019; Nayak et al., 2016). As can be noted in Figure 4.7, a significant decrease ($P < 0.05$) in relative fluorescence was shown by PP-Z either under UV-A light

(-4.6 ± 0.8) or in the dark (-3.7 ± 0.2) as compared to the fluorophore solution alone under UV-A light. This confirms that UV-A light by itself doesn't generate ROS and that is harmless against *E. coli* K12. A report found similar results when determining the fluorescence intensity of a fluorometric probe exposed to UV-A light when compared to the probe in the dark in the presence of gallic acid. They found that there was no significant ($P > 0.05$) difference between the two treatments (Q. Wang et al., 2017). Ding et al. also confirmed this finding when they tested fluorescence intensity in *E. coli* O157:H7 in the presence benzoic acid (Ding, Alborzi, Bastarrachea, & Tikekar, 2018). However, the values of relative fluorescence shown by PP-Z either in the dark or under UV-A light (although different) were not significantly different ($P > 0.05$). This suggests that other antimicrobial mechanisms may be involved, such as protonated amine groups that interact with the Ca^{2+} ions in the cell wall and this leads to loss of fluidity, which may relate to the charged nature of PP-Z after being protonated (Deka et al., 2015).

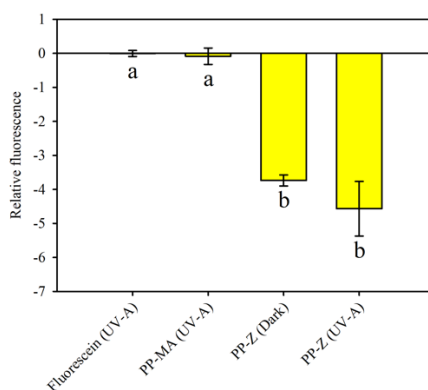


Figure 4.7 Fluorometric evaluation of ROS generation. Treatments that share the same lowercase letter are not significantly different ($P > 0.05$).

Cell Morphology Analysis by SEM

Figure 4.8 displays an evident change was observed between the control *E. coli* K12 cells (kept in the dark) and those exposed to the combination of PP-Z and UV-A light. The control cells exhibited the characteristic *E. coli* bacillar shape. In contrast, the cells exposed to PP-Z and UV-A light exhibited damaged cell structure and what appeared to be leakage of cell components. It is possible that zein works in a similar mechanism polycations do in that they interact with the cell wall cell membrane through ionic interactions, causing disruption on their structure and functionality (Bastarrachea, Denis-Rohr, et al., 2015; Carmona-Ribeiro & de Melo Carrasco, 2013).

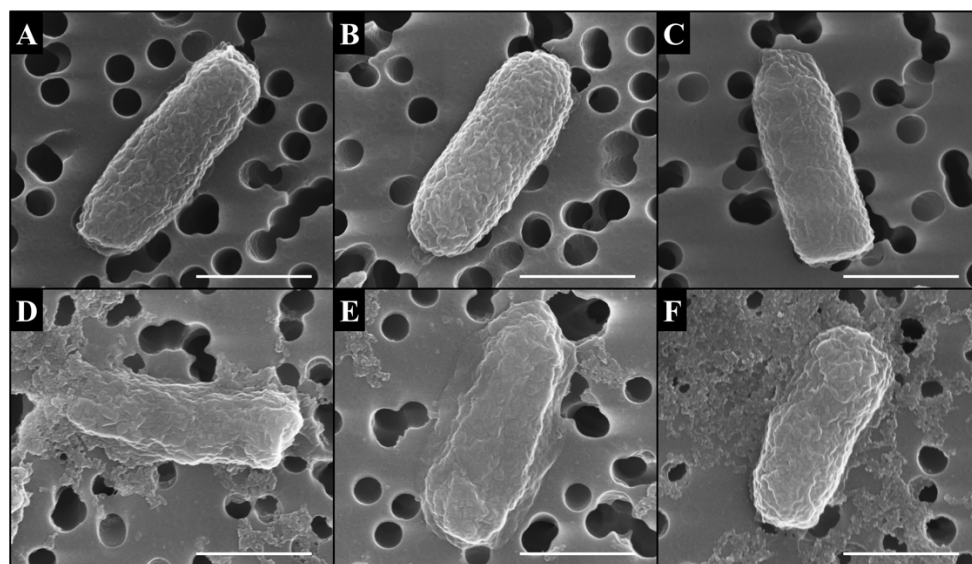


Figure 4.8 Cell morphology of control *E. coli* K12 cells (A-C) and cells exposed to PP-Z under UV-A light (D-F). Scale bars represent 1 μm .

CONCLUSIONS

In the present work, it has been confirmed that the protein zein (a substance that is not inherently antimicrobial) can be used to fabricate stable and reusable UV light-activated antimicrobial materials. PP-Z demonstrated to be able to reduce the *E. coli* K12

population in aqueous solutions by ~ 90 (1 logarithmic reduction) for at least 10 cycles under UV-A light. Fluorometric evaluations suggested that its antimicrobial mechanism may be driven by ROS generation and disruption of microbial cell structure. Microscopic and spectroscopic techniques also confirmed marginal changes in the chemical and physical integrity of PP-Z under continuous usage and lack of fouling or cell attachment. This concept could represent an attractive option to prevent or reduce microbial contamination in the food industry (as a processing or packaging aide), or in the biomedical field. It could also offer an alternative for recycling of plastic waste. Future work could involve optimizing its antimicrobial power by tuning the surface area of available protein in the modified material.

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CHAPTER 5

OVERALL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

Chitosan-modified polypropylene showed antimicrobial effectiveness across 10 cycles of reusability evaluations. It was able to reduce bacterial growth by $94.0 \pm 3.3\%$ across the 10 cycles. Fluorometric assays suggested this may have been due to the generation of ROS. This assay showed ROS being generated by the chitosan material in the dark and under UV-A light. This indicates that the polycation is able to generate ROS even in the dark. The antimicrobial assay also showed that the chitosan material left in the dark was able to reduce the microbial load, but not nearly as much as the material exposed to UV-A light. These results suggest the UV-A light may enhance the antimicrobial effectiveness of the modified material. SEM images of the bacterial cells also seemed to confirm this phenomenon. The chitosan material also demonstrated stability after the 10 cycles. ATR-FTIR and SEM of the surface of the material confirm that the surface chemistry and physical material changed very little. This material has proven to be a robust and effective antimicrobial aid against *E. coli* K12.

Much of what has been said of chitosan may be attributed to the zein-modified polypropylene. It was not quite as effective across the 10 cycles of reusability as it only reduced bacterial growth by $89.3 \pm 3.6\%$ as compared to $94 \pm 3.3\%$ from the chitosan-modified material. This is still promising as zein is not an inherent antimicrobial. This study has shown that it may have possibilities as an antimicrobial aid. Analysis of the surface confirmed that this material is also stable after the 10 cycles.

Figure 5.1 below presents the colony forming units per mL (CFU/mL) in relation to the fluorescence in the presence of each material under a certain treatment. As can be

seen there is a significant difference ($P < 0.05$) between the CFU/mL of the PP-MVE-CHI in the dark and the CFU/mL of the PP-MVE-CHI under UV-A light. As can also be seen is the lower amount of fluorescence in the PP-MVE-CHI treated to UV-A light. Although it is not significant, this suggests that more fluorophore probe is being degraded when exposed to the PP-MVE-CHI material and UV-A light. This may mean more ROS are being generated. As was mentioned above, the ROS being generated may be why there is less CFU/mL in the presence of PP-MVE-CHI under UV-A light. Figure 5.1 also shows a similar trend for the PP-Z materials. The CFU/mL is not significantly different between the two PP-Z conditions, but the PP-Z exposed to UV-A light is lower. The fluorescence is also lower within that same treatment. This again suggests that more ROS are being generated when exposed to the material and UV-A light, which in turn may limit the amount of bacterial growth.

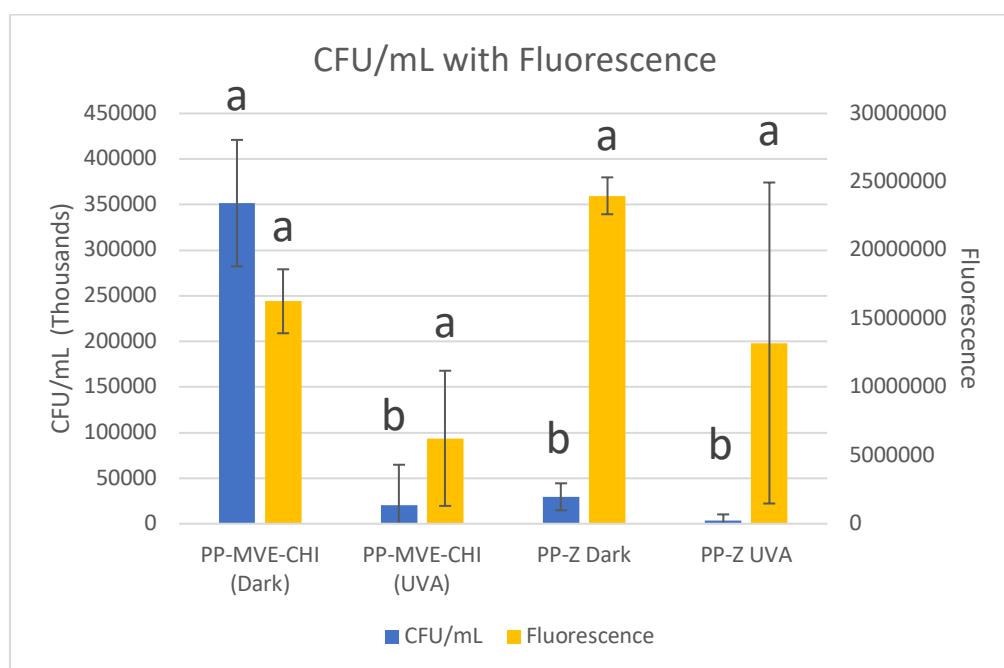


Figure 5.1 CFU/mL results compared with fluorescence for PP-MVE-CHI treatments and PP-Z treatments. Treatments within the same category (CFU/mL or Fluorescence) that share the same letter are not significantly different ($P > 0.05$).

Further research may involve testing these materials against other microorganisms, particularly Gram-positive bacteria. This may help determine how wide the spectrum is for these antimicrobial agents. Other research may also include testing these materials in the presence of organic substances (the studies herein were conducted in aqueous environments). Further analysis may include better understanding the mode of action for both of the aforementioned materials. This would involve determining specific ROS being generated and if this is the primary mechanism as compared to DNA disruption, which can be caused by certain wavelengths of UV light. Another analysis would be to determine the role of pH and protonation of the materials. To do so the modified materials would be tested in both acidic and basic solutions.

APPENDICES

APPENDIX A

FOR CHAPTER 3



Antimicrobial Light-Activated Polypropylene Modified with Chitosan: Characterization and Reusability

Author: Andrew T. Gagon, David W. Britt, Luis J. Bastarrachea

Publication: Journal of Agricultural and Food Chemistry

Publisher: American Chemical Society

Date: Dec 1, 2019

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APPENDIX B

FOR CHAPTER 4



Zein-modified antimicrobial polypropylene: Characterization and reusability upon UV-A light exposure

Author: Andrew T. Gagon, David W. Britt, Luis J. Bastarrachea

Publication: LWT - Food Science and Technology

Publisher: Elsevier

Date: March 2020

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